CIRCULATING SEX HORMONE LEVELS IN RATS: A SYSTEMATIC ANALYSIS OF THE LITERATURE AND PERINATAL BPA STUDY

BY

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THESIS

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Abstract

In the study of reproductive toxicology, animal models play an important role in elucidating the mechanisms by which a toxicant exerts its deleterious effects, as well as serving as a translational system for comparison to human biology. The anatomy, physiology, and molecular mechanisms of the reproductive system of these models should first be well characterized within the species prior to making inter-species predictions on the toxicant's effects. Here we conducted a systematic analysis of the available literature to characterize within the rat model how three different, popular outbred strains (Wistar, Sprague-Dawley, and Long-Evans) may each possess distinct levels of basal peripheral testosterone (T), estradiol (E2), or progesterone (P4) levels, according to rat sex and age. We found that estradiol and testosterone, but not progesterone, were different depending on rat strain, which could possibly point to inherent differences in the hormonal regulatory systems between strains, and also potential differences between strains in susceptibility to the detrimental effects of reproductive toxicants. As an extension of this analysis of the factors that influence sex hormones, we also examined the effect of the known endocrine disrupting chemical, bisphenol-A (BPA), at environmentally relevant ranges on the sex hormones, follicle-stimulating hormone (FSH), luteinizing hormone (LH), T, E2, and P4 in perinatally exposed, infantile rats. We found no effect of BPA on sex hormone levels in these rats, which is in both concordance and opposition to other studies that have examined this developmental time point. The current contradictory nature of the literature regarding the effect of environmentally relevant dosages of BPA could benefit from an examination of the factors other than toxicant exposure leading to the different results.

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Thesis Introduction

The Study of Reproductive Toxicology

The academic discipline of reproductive toxicology has been defined as the study of chemicals and agents on the reproductive system and the identification of adverse effects that interfere with the process of successful and healthy reproduction (Hood 2011). The field studies the disruptive effects of exogenous substances on the reproductive physiology of both sexes, and is intimately intertwined with the fields of teratology, developmental biology, and endocrinology. Such chemicals that are specifically identified as harmful to reproductive functioning are then subjected to experimentation to identify the mechanisms by which they might exert their deleterious effects. These effects typically will be noticeable at the gonadal level, and consequently, also at the hypothalamic and pituitary levels due to the functional association of the three tissues in the hypothalamic-pituitary-gonadal (HPG) axis (Peretz, et al., 2011). In addition to the gonad and the major axis that controls them, reproductive toxicologists can also focus on the accessory sex organs: the oviducts, uterus, vagina, and mammary glands in the female, and the epididymis, seminal vesicles, and prostate in the male. Harmful effects on fertility are also evaluated beginning with sexual behavior, conception, and continuing throughout pregnancy and eventually parturition (Peretz, et al., 2011). The more recent interest in reproductive toxicology even extends into transgenerational effects in offspring (Skinner 2007; Skinner, et al., 2010).

The Role of Animal Models in Reproductive Toxicology

Although new *in vitro* molecular and cellular methods have continued to emerge and advance in recent years, the reality of *in vivo* animal testing will likely persist due to the need for whole-system assessment of a toxicant's effects (Hood, 2006). Laboratory bred animals have served this purpose to extrapolate findings and comparatively explain human processes. An animal model is chosen based on the specific requirements of a research question and/or study design. The general consensus in reproductive toxicology is that the animal model chosen for primary screening of a chemical should (1) have a maternal-placental-fetal relationship similar to humans, and thus mammals are preferable, (2) have a short gestation period, with a large litter size, and easy mode of breeding, and (3) be economically easy to house and technically easy to handle and collect samples from (Kacew 2001; Schardein, et al., 1985).

Thus, animal models in reproductive toxicology have traditionally involved rodents — usually mice or rats—, but also rabbits, dogs, and some non-human primates (Schardein, et al., 1985); large animals with prolonged gestation are only usually used in cases where the issue of teratogenicity needs to be examined in more detail. Invertebrates, fish, amphibians, and birds are usually utilized mainly for the recognition of ecotoxicity, rather than for comparison to human physiology as they have demonstrated a greater number of differences in their responses to toxicant exposure than when comparing humans to other mammals (Touart 2006). Nonetheless, the zebrafish, *Xenopus laevis*, and the chicken have all been invaluable models that have facilitated the study of early embryonic development (Oelgeschläger 2014).

However, no one species shares entirely similar traits to humans, and thus is best suited for comparison as noted by Amann (1982). Since the degree of control is one of the defining

aspects of any good study, an animal model should be chosen for specific reasons relating to the objective of the experimental hypothesis. As an example, rats are usually preferred to mice and hamsters for their augmented intelligence and organ size, and as a popular animal for toxicology studies historically, their reproductive characterization is well documented (Gray, et al., 2010; Hedrich, 2000; Kacew, 2001; Weber, et al., 2010). The rat model has been used for decades in reproductive toxicology and other biological sciences. Thus, a plethora of phenotypic reference information is available through databases such as the Rat Genome Database (RGD), which has consolidated a variety of physiologic measurements that can be searched based on the type of clinical measurement, experimental condition, measurement method, or strain (Laulederkind, et al., 2013). However, the rat is not an ideal candidate for longitudinal study of semen or spermatozoa (Amann, 1982). Mice and rats are known to be different from humans in several aspects of the process of implantation: such as the ability to delay implantation after fertilization, and the rapid speed at which the process occurs, making it a difficult model for the study of early embryonic events (Lee & DeMayo, 2004). Additionally, measuring fertility by the ability to produce offspring is an endpoint that is not nearly as sensitive in rodents as in humans, since rodents normally have large litter sizes and are robustly fertile compared to humans (Seed, et al., 1996). Observing the early mechanisms of implantation may be better suited to animals with a prolonged apposition and attachment phase, a characteristic of epitheliochorial placentaed mammals like cows, pigs, and sheep. However, rodents present numerous practical advantages as animal models in general (e.g., similar metabolic pathways, similar anatomical and physiological characteristics, small size, short life span) and in reproductive toxicology specifically: e.g., short gestation time and large litter size (Kacew, 2001). In summary, it is

important to utilize in-depth knowledge of the physiology of each species, and the known species homologies or divergences, in any discussion about the absence or presence of toxicological effects in studies using animal models. It is also important to understand these intra- and interspecies characteristics when comparing studies.

Sex Hormones

Steroidal sex hormones

The sex hormones consist of the steroidal gonadal hormones, as well as several non-steroidal peptide hormones derived from the anterior pituitary. The gonadal steroid hormones are usually general classified as androgens, estrogens, and progestrogens. Androgens (derived from the Greek root "andro" that means "male") are typically associated with male features because of their key role in regulating the functions of the primary sex organs and the development of secondary sex characteristics specific to males. The most prominently discussed androgen, testosterone (T), is known to promote the development of the testis, prostate, sperm, body hair, and anabolic accumulation of muscle and bone mass, and was found to be approximately ten times higher in adult men than women (Boulpaep, et al., 2009; Mooradian, et al., 1987; Taieb, et al., 2003). Conversely, estrogens are associated with female sexual characteristics. The major estrogen, 17β-estradiol (estradiol or E2), is essential to maintaining the tissues and secretions of the female reproductive system, and especially the lining of the uterus as part of the estrous or menstrual cycle (Boulpaep, et al., 2009). 17β-estradiol also promotes the development of female secondary sex characteristics by affecting bone development during puberty and site-specific fat deposition leading to the formation of breasts

(Belun 2014; Boulpaep, et al., 2009). Despite the reputation of estradiol as a “female hormone”, it also plays an important role in males, such as in the early events of neuronal sexual differentiation during fetal and neonatal development by masculinizing specific nuclei within the hypothalamus (McCarthy, 2008). Progesterone, the major progestrogen, is secreted in high amounts by the female corpus luteum, and induces cell differentiation and secretions of the uterine endometrium during the estrous cycle. It is also critical for implantation of the fertilized embryo and the maintenance of pregnancy (Kim, et al., 2013). The role of progesterone in the male is less understood, though it has been suggested that it plays a role during development on the sexual dimorphic regions of the brain (Wagner, 2006).

After being studied for decades, the cell signaling associated with steroidal sex hormones is relatively well defined (McKenna, 2015). Testosterone primarily binds to the nuclear receptor androgen receptor (AR). The androgen receptor is expressed by a wide variety of tissues, but is most present and influential in reproduction-related areas in the brain (e.g., the medial preoptic and ventromedial nuclei), prostate, seminal vesicles, testes, ovaries, and uterus (Dart and Alwyn, 2013, De Winter, et al., 1991; Simerly, et al., 1990). Estradiol binds to nuclear-located estrogen receptors alpha and beta (ERα and ERβ, also known as ESR1 and ESR2). These two types of classical estrogen receptor are widely expressed but in different ratios depending on the tissue (Couse, et al., 1997; Kuiper, et al., 1997; Deroo & Korach, 2006). The progesterone receptor (PR) is also a member of the nuclear steroid-receptor superfamily. Progesterone receptors are also ubiquitously expressed in a variety of non-reproductive and reproductive tissue, including the uterus, brain, ovary, and testes (Graham & Clarke, 1997; Scarpin, et al., 2009).

Peptide sex hormones

The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are glycoproteins that are released from the gonadotroph neurons in the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) released from the hypothalamus. Both LH and FSH are in the same family of glycoproteins as thyroid-stimulating hormone (TSH), all of which share the same alpha domain, while the beta domain defines their receptor-binding specificity (Boulpaep, et al., 2009).

The primary action of LH in both sexes, is to stimulate the production of cholesterol side chain cleaving enzyme, the first enzyme of the steroidogenic pathway in which cholesterol is converted into the various steroid hormones. LH regulates androstenedione production in the theca cells of the ovary, the precursor to both testosterone and estradiol (Belun & Adashi 2011). FSH exerts its indirect influence on steroidogenesis by promoting female ovarian follicle growth and recruitment, as well as direct influence within the pathway by stimulating aromatase activity within the granulosa cells (Erickson and Hsueh 1978).

In females, LH binds to its corresponding receptor integrated within the cell membranes of the steroid-secreting ovarian follicular cells: both granulosa and theca cells, as well as luteal cells during the estrous or menstrual cycle (Ascoli & Fanelli 2002, Boulpaep, et al., 2009). The FSH receptor is primarily expressed within the membranes of granulosa cells (Simoni, et al., 1997, Boulpaep, et al., 2009). It regulates estradiol production and the growth of ovarian follicles. Both FSH and LH together are required to produce estradiol during steroidogenesis in the female because no one cell type, theca or granulosa cell, is able to produce progesterone on its own (Boulpaep, et al., 2009; Magoffin, 2005). In males, LH receptors are located on the

steroid-secreting testicular Leydig cells, whereas the role of FSH is mainly on the Sertoli cells, which regulate spermatogenesis (Asatiani, et al., 2002, Boulpaep, et al., 2009).

The hypothalamic-pituitary-gonadal axis

The regulatory relationship between steroid sex hormones and glycoprotein gonadotropins (FSH and LH) is the basis for the hypothalamic-pituitary-gonadal axis. The production of steroid hormones by the gonads is stimulated by glycoprotein gonadotropins. The steroid hormones in turn feedback to both the hypothalamus and pituitary gland to down-regulate the production of the gonadotropins. This hormonal regulation loop develops in a step-wise fashion from birth until puberty (Boulpaep, et al., 2009).

Sex hormone variability factors

In addition to intrinsic physiological mechanisms of sex hormone regulation, external factors are known to affect hormone levels. Some factors are endogenous: e.g., sleep-wake diurnal patterns (Winters 1991; Tenover, et al., 1988; Reinberg & Lagoguy 1978; Reinberg, et al., 1975; Plymate, et al., 1989; Nicolau, et al., 1984a,b; Montanini, et al., 1988; Lévi, et al., 1988; Faiman & Winter 1971; Diver, et al., 2003; de la Torre, et al., 1981; Cooke, et al., 1993; Bremner, et al., 1983), pregnancy (Kacew 2001), psychogenic or physical stress (Belun & Adashi 2011, Rivier & Rivest 1991), or amount of body fat (Tchernof & Despres 1999). Other factors are exogenous: light-dark exposure (Critser, et al., 1987, Fantie, et al., 1984), endocrine disrupting chemicals (Diamanti-Kandarakis, et al., 2009; Gore, et al., 2015; Gupta, et al., 2010; Peretz, et al.,

2011; Sharara, et al., 1998), hormone-supplementation regimens, and some therapeutic pharmacological agents (Geisler, et al., 2002; Mattson & Cramer 1985; Merkatz, et al., 1993).

Reference Information for Sex Hormones

Due to the widespread influence that sex hormones have on physiology, and their foremost role in regulation of reproductive function and development, their levels as measured in circulation constitute an important endpoint in many reproductive toxicology studies (Goldman, et al., 2007, Melnick, et al., 2002). However, the inclusion of sex hormone levels is sometimes listed as an optional endpoint in experimental guidelines, likely due to concerns over assay sensitivity and accuracy, and inherent physiological difficulties in obtaining reproducible or representative measurements (Holmes, et al., 1998; McMaster, et al., 2001; Rosner, et al., 2007).

Ideally, any blood chemistry measurement should be compared to a measurement from a control sample taken from within the same individual. However, this is not always feasible due to the constraint of time and small blood volume of common laboratory animals. Therefore, other control methods have been accepted as reasonable, such as a separate untreated group, vehicle-treated group, or sham-operated group in studies involving a surgery, with these controls undergoing the full extent of the experimental conditions at the same time as the chemically-treated group (Johnson & Besselsen 2002). In addition to control groups, reference intervals for blood chemistry are also useful. Such ranges are often used in the clinical setting for pathological determination and can be used to estimate the test sensitivity that will be required to measure sex hormone levels in new samples from a specific study. Reference intervals, in

either research or clinics, should also be established internally within the laboratory that is to be performing the procedure whenever possible (Jones & Barker 2008, Jung & Adeli 2009). Especially in cases of sex hormones, which are highly variable as a result of numerous factors, the establishment of a normal range of values based on healthy individuals can assist in determining whether any particular laboratory finding is of interpretable value by first determining if the measured hormone level will have the accuracy, precision, and concordance with respect to these factors.

Gender-, age-, and cycle- specific reference intervals of sex hormone levels have been described extensively for humans, although generally in healthy populations (Ukkola, et al., 2001; Eskelinen, et al., 2007; Schüring, et al., 2016; Elmlinger, et al., 2002; Bhasin, et al., 2011; Kishnir, et al., 2010; Soldin, et al., 2005, Milewicz, et al., 2013). There are more limited data available for mice (McNamara, et al., 2010). Although the Rat Genome Database is an excellent tool for the organization of published studies reporting other blood chemistry data, the amount of information on sex hormone levels in the database shows high variability in terms of concentration values from less than ten small individual studies that used different methodology, and collectively included a small number of strains and age points (Laulederkind, et al., 2013).

Thesis Goals

The aspects of this thesis are two-fold. First, this thesis project was designed to investigate the conceptual features of the study of reproductive toxicology, specifically the role of sex hormones as an experimental endpoint, and the study design characteristics on which

their measurement values are so heavily dependent. Based on all of the above considerations, I first evaluated one possible determinant of sex hormone levels—strain—in a popular animal model, the rat, by conducting an analysis of the available literature to evaluate whether there was an observable difference in steroid sex hormone measurements between strains of rat. Secondly, I performed a simple toxicological assessment of the endocrine disrupting chemical, bisphenol-A (BPA) on the hormone levels of developing rats. Overall, this thesis demonstrates the importance of commonly overlooked conceptual elements that should be considered when designing a reproductive toxicology study.

References

Amann, R. P. (1982). Use of animal models for detecting specific alterations in reproduction. *Fundamental and Applied Toxicology*, *2*(1), 13-26.

Asatiani, K., Gromoll, J., Eckardstein, S. V., et al., (2002). Distribution and function of FSH receptor genetic variants in normal men. *Andrologia*, *34*(3), 172-176.

Ascoli, M., Fanelli, F., Segaloff, D. L. (2002). The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocrine Reviews*,*23*(2), 141-174.

Bhasin, S., Pencina, M., Jasuja, G. K., et al., (2011). Reference ranges for testosterone in men generated using liquid chromatography tandem mass spectrometry in a community-based sample of healthy nonobese young men in the Framingham Heart Study and applied to three geographically distinct cohorts. *The Journal of Clinical Endocrinology & Metabolism*, *96*(8), 2430-2439.

Boulpaep, E. L., Boron, W. F., Caplan, M. J., et al., (2009). Medical Physiology a Cellular and Molecular Approach. *Signal Transduction*, *48*, 27.

Bremner, W. J., Vitiello, M. V., Prinz, P. N. (1983). Loss of Circadian Rhythmicity in Blood Testosterone Levels with Aging in Normal Men. *The Journal of Clinical Endocrinology & Metabolism*, *56*(6), 1278-1281.

Bulun, S. E. (2014). Aromatase and estrogen receptor α deficiency. *Fertility and sterility*, *101*(2), 323-329.

Bulun, S. E., Adashi, E. Y. (2011). The physiology and pathology of the female reproductive axis. *Williams Textbook of Endocrinology. 12th ed. Philadelphia, PA: Elsevier Saunders*.

Cooke, R. R., McIntosh, J. E. A., McIntosh, R. P. (1993). Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model. *Clinical endocrinology*, *39*(2), 163-171.

Couse, J. F., Lindzey, J., Grandien, et al., (1997). Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. *Endocrinology*, *138*(11), 4613-4621.

Critser, J. K., Block, T. M., Folkman, S., et al., (1987). Effect of photoperiod on LH, FSH, prolactin and melatonin patterns in ovariectomized prepubertal heifers. *Journal of Reproduction and Fertility*, *81*(1), 29-39.

Dart, D. A., Waxman, J., Aboagye, E. O., et al., (2013). Visualising androgen receptor activity in male and female mice. *PLoS One*, *8*(8), e71694.

de la Torre, B., Sjöberg, B., Hedman, M., et al., (1981). A study of the short-time variation and interrelationship of plasma hormone levels reflecting pituitary, adrenocortical and testicular function in fertile men. *International Journal of Andrology*, *4*(1-6), 532-545.

Deroo, B. J., Korach, K. S. (2006). Estrogen receptors and human disease. *The Journal of Clinical Investigation*, *116*(3), 561-570.

De Winter, J. R., Trapman, J., Vermey, M., et al., (1991). Androgen receptor expression in human tissues: an immunohistochemical study. *Journal of Histochemistry Cytochemistry*, 39(7), 927-936.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., et al., (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*, *30*(4), 293-342.

Diver, M. J., Imtiaz, K. E., Ahmad, A. M., et al., (2003). Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clinical Endocrinology*, *58*(6), 710-717.

Elmlinger, M. W., Kühnel, W., Ranke, M. B. (2002). Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults. *Clinical Chemistry and Laboratory Medicine*, *40*(11), 1151-1160.

Enders, A. C. (1999). Trophoblast-uterine interactions in the first days of implantation: models for the study of implantation events in the human. *Seminars in reproductive medicine* (Vol. 18, No. 3, pp. 255-263).

Erickson, G. F., Hsueh, A. J. W. (1978). Stimulation of Aromatase Activity by Follicle Stimulating Hormone in Rat Granulosa Cells in Vivo and in Vitro*. *Endocrinology*, *102*(4), 1275-1282.

Eskelinen, S., Vahlberg, T., Isoaho, R., et al., (2007). Biochemical reference intervals for sex hormones with a new AutoDelfia method in aged men. *Clinical Chemical Laboratory Medicine*, *45*(2), 249-253.

Faiman, C., Winter, J. S. D. (1971). Diurnal Cycles in Plasma FSH, Testosterone and Cortisol in Men. *The Journal of Clinical Endocrinology & Metabolism*, *33*(2), 186-192.

Fantie, B. D., Brown, R. E., Moger, W. H. (1984). Constant lighting conditions affect sexual behaviour and hormone levels in adult male rats. *Journal of Reproduction and Fertility*, *72*(2), 435-441.

Geisler, J., Haynes, B., Anker, G., et al., (2002). Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. *Journal of Clinical Oncology*, *20*(3), 751-757.

Goldman, J. M., Murr, A. S., Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *80*(2), 84-97.

Gore, A. C., Chappell, V. A., Fenton, S. E., et al. (2015). EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocrine reviews, 36(6), E1-E150.

Graham, J. D., Clarke, C. L. (1997). Physiological action of progesterone in target tissues 1. *Endocrine reviews*, *18*(4), 502-519.

Gray, L. E., Ryan, B., Hotchkiss, A. K., et al., (2010). Rebuttal of "flawed experimental design reveals the need for guidelines requiring appropriate positive controls in endocrine disruption research" by vom Saal.*Toxicological Sciences*, *115*(2), 614-620.

Gupta, R. K., Singh, J. M., Leslie, T. C., et al. (2010). Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. *Toxicology and applied pharmacology*, *242*(2), 224-230.

Hedrich, H. J. (2000). History, strains and models. *The Laboratory Rat. Academic Press, London*, 3-8.

Holmes, P., Humfrey, C., Scullion, M., et al., (1998). Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals Capable of Affecting the Reproductive Process. *Prepared by the MRC Institute for Environment and Health for the UK Department of Environment, Transport and the Regions http://www. oecd. org/dataoecd/58/27/1898464.pdf*.

Hood, R. D. (2006). Principles of developmental toxicology revisited. *Developmental and reproductive toxicology: A practical approach*, 3-14.

Hood, R. D. (Ed.). (2011). *Developmental and reproductive toxicology: a practical approach*. CRC Press.

Johnson, P. D., Besselsen, D. G. (2002). Practical aspects of experimental design in animal research. *ILAR Journal*, *43*(4), 202-206.

Jones G, Barker A. Reference Intervals. *The Clinical Biochemist Reviews*. 2008;29(Suppl 1):S93-S97.

Jung, B., Adeli, K. (2009). Clinical laboratory reference intervals in pediatrics: the CALIPER initiative. *Clinical biochemistry*, *42*(16), 1589-1595.

Kacew, S. (2001). Confounding factors in toxicity testing. *Toxicology*, *160*(1), 87-96.

Kim, J. J., Kurita, T., Bulun, S. E. (2013). Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocrine Reviews*, *34*(1), 130-162.

Kuiper, G. G., Carlsson, B. O., Grandien, K. A. J., et al., (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β.*Endocrinology*, *138*(3), 863-870.

Kushnir, M. M., Blamires, T., Rockwood, A. L., et al., (2010). Liquid chromatography–tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clinical Chemistry*,*56*(7), 1138-1147.

Laulederkind, S. J., Hayman, G. T., Wang, S. J., et al., (2013). The Rat Genome Database 2013—data, tools and users. *Briefings in bioinformatics*, *14*(4), 520-526.

Lee, K. Y., DeMayo, F. J. (2004). Animal models of implantation. *Reproduction*, *128*(6), 679-695.

Lévi, F. A., Canon, C., Touitou, Y., et al., (1988). Circadian rhythms in circulating T lymphocyte subtypes and plasma testosterone, total and free cortisol in five healthy men. *Clinical and Experimental Immunology*, *71*(2), 329.

Magoffin, D. A. (2005). Ovarian theca cell. The international journal of biochemistry & cell biology, 37(7), 1344-1349.

Mattson, R. H., Cramer, J. A. (1985). Epilepsy, sex hormones, and antiepileptic drugs. *Epilepsia*, *26*(s1), S40-S51.

McCarthy, M. M. (2008). Estradiol and the developing brain. *Physiological Reviews*, *88*(1), 91-134.

McKenna, N.J. (2015). Gonadal Steroid Action. *Knobil and Neill's Physiology of Reproduction*, *4*, 313-329.

McMaster, M. E., Jardine, J. J., Ankley, G. T., et al., (2001). An interlaboratory study on the use of steroid hormones in examining endocrine disruption. *Environmental toxicology and chemistry*, *20*(9), 2081-2087.

McNamara, K. M., Harwood, D. T., Simanainen, U., et al., (2010). Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography–tandem mass spectrometry. *The Journal of Steroid Biochemistry and Molecular Biology*,*121*(3), 611-618.

Melnick, R., Lucier, G., Wolfe, M., et al., (2002). Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environmental health perspectives*, *110*(4), 427.

Merkatz, R. B., Temple, R., Sobel, S., et al., (1993). Women in Clinical Trials of New Drugs--A Change in Food and Drug Administration Policy. *New England Journal of Medicine*, *329*(4), 292-296.

Milewicz, A., Krzyżanowska-Świniarska, B., Miazgowski, T.,et al., (2013). The reference values of sex hormones and SHBG serum levels in subjects over 65 years old—The PolSenior Study. *Endokrynologia Polska*, *64*(2), 82-93.

Mooradian, A. D., Morley, J. E., Korenman, S. G. (1987). Biological actions of androgens. *Endocrine Reviews*, *8*(1), 1-28.

Montanini, V., Simoni, M., Chiossi, G., et al., (1988). Age-related changes in plasma dehydroepiandrosterone sulphate, cortisol, testosterone and free testosterone circadian rhythms in adult men. *Hormone Research in Paediatrics*, *29*(1), 1-6.

Nicolau, G. Y., Lakatua, D., Sackett-Lundeen, L., et al., (1984b). Circadian and circannual rhythms of hormonal variables in elderly men and women. *Chronobiology international*, *1*(4), 301-319.

Nicolau, G. Y., Haus, E., Lakatua, D. J., et al., (1984a). Circadian and circannual variations of FSH, LH, testosterone, dehydroepiandrosterone-sulfate (DHEA-S) and 17-hydroxy progesterone (17 OH-Prog) in elderly men and women. *Endocrinologie*, *23*(4), 223-246.

Oelgeschläger, M. (2014, May 16). *Non-mammalian animal models in developmental toxicology*. Retrieved June 7, 2016, from http://www.bfr.bund.de/cm/349/non-mammalian-animal-models-in-developmental-toxicology.pdf

Peretz, J., Craig, Z. R., Bunting, S., et al., (2011). Experimental approaches to evaluate mechanisms of reproductive toxicity. In R. D. Hood (Ed.), *Developmental and Reproductive Toxicology: A Practical Approach, Third Edition* (pp. 45-59). CRC Press.

Plymate, S. R., Tenover, J. S., Bremner, W. J. (1989). Circadian variation in testosterone, sex hormone-binding globulin, and calculated non-sex hormone-binding globulin bound testosterone in healthy young and elderly men. *Journal of Andrology*, *10*(5), 366-371.

Reinberg, A., Lagoguey, M., Chauffournier, J. M., et al., (1975). Circannual and circadian rhythms in plasma testosterone in five healthy young Parisian males. *Acta Endocrinologica*, *80*(4), 732-734.

Reinberg, A., Lagoguey, M. (1978). Circadian and circannual rhythms in sexual activity and plasma hormones (FSH, LH, testosterone) of five human males. *Archives of Sexual Behavior*, *7*(1), 13-30.

Rosner, W., Auchus, R. J., Azziz, R., et al. (2007). Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. The Journal of Clinical Endocrinology & Metabolism, 92(2), 405-413.

Rivier, C., Rivest, S. (1991). Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biology of Reproduction*, *45*(4), 523-532.

Scarpin, K. M., Graham, J. D., Mote, P. A., et al., (2009). Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. *Nucl Recept Signal*, *7*(5), 459.

Schardein, J. L., Schwetz, B. A., Kenel, M. F. (1985). Species sensitivities and prediction of teratogenic potential. *Environmental health perspectives*,*61*, 55.

Schüring, A. N., Kelsch, R., Pierściński, G., et al., (2016). Establishing Reference Intervals for Sex Hormones on the Analytical Platforms Advia Centaur and Immulite 2000XP. *Annals of Laboratory Medicine*, *36*(1), 55-59.

Seed, J., Chapin, R. E., Clegg, E. D., Dostal, L. A. (1996). Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. *Reproductive Toxicology*, *10*(3), 237-244.

Sharara, F. I., Seifer, D. B., Flaws, J. A. (1998). Environmental toxicants and female reproduction. Fertility and sterility, 70(4), 613-622.

Simerly, R. B., Swanson, L. W., Chang, C., et al., (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *Journal of Comparative Neurology*,*294*(1), 76-95.

Simoni, M., Gromoll, J., Nieschlag, E. (1997). The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology 1. *Endocrine Reviews*, *18*(6), 739-773.

Skinner, M. K. (2007). Endocrine disruptors and epigenetic transgenerational disease etiology. *Pediatric research*, *61*, 48R-50R.

Skinner, M. K., Manikkam, M., Guerrero-Bosagna, C. (2010). Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology Metabolism*, *21*(4), 214-222.

Soldin, O. P., Hoffman, E. G., Waring, M. A., et al., (2005). Pediatric reference intervals for FSH, LH, estradiol, T3, free T3, cortisol, and growth hormone on the DPC IMMULITE 1000. *Clinica Chimica Acta*, *355*(1), 205-210.

Taieb, J., Mathian, B., Millot, F., et al., (2003). Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography–mass spectrometry in sera from 116 men, women, and children. *Clinical chemistry*, *49*(8), 1381-1395.

Tchernof, A., Despres, J. P. (1999). Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women. *Hormone and metabolic research= Hormon-und Stoffwechselforschung= Hormones et Metabolisme*, *32*(11-12), 526-536.

Tenover, J. S., Matsumoto, A. M., Clifton, D. K., et al., (1988). Age-related alterations in the circadian rhythms of pulsatile luteinizing hormone and testosterone secretion in healthy men. *Journal of Gerontology*, *43*(6), M163-M169.

Touart, L. (2006). OECD Environment, Health and Safety Publications Series on Testing and Assessment No. XX.

Ukkola, O., Gagnon, J., Rankinen, T., et al., (2001). Age, body mass index, race and other determinants of steroid hormone variability: the HERITAGE Family Study. *European Journal of Endocrinology*, *145*(1), 1-9.

Wagner, C. K. (2006). The many faces of progesterone: a role in adult and developing male brain. *Frontiers in neuroendocrinology*, *27*(3), 340-359.

Weber, K., Razinger, T., Hardisty, J. F., et al., (2011). Differences in rat models used in routine toxicity studies. *International journal of toxicology*, *30*(2), 162-173.

Winters, S. J. (1991). Diurnal rhythm of testosterone and luteinizing hormone in hypogonadal men. *Journal of Andrology*, *12*(3), 185-190.

Chapter 1: A Systematic Analysis of the Literature to Evaluate the Effect of Strain on Peripheral Sex Hormone Levels in Rats

Introduction

The laboratory rat has served an important historical role in biomedical and psychological research and continues as a widely used animal model of human physiology. The similarity of many anatomical and physiological traits to humans, small size, short gestation period, intelligence, manageable temperament, and economic feasibility, make the rat a choice research model in a wide variety of disciplines. The amount of information on the characteristics of various strains and stocks is also heavily documented and available in numerous databases for use by researchers.

The most commonly used rats in research, including generically named stocks, Wistar, Sprague-Dawley, Long-Evans, Holtzmann, Albany, among others, are all derived from an original randomly bred (outbred) stock of the Norway rat (*Rattus norvegicus*) (Hedrich 2000), and are named for their historical origin more than for similar genotypic or phenotypic characteristics. These commercially available stocks may be subdivided into colony designations within their breeding facilities, although there is high likelihood of genetic variability when comparing colony to colony due to less stringent breeding practices than inbred lines (Kacew & Festing 1999). Although typically more expensive, inbred lines are desirable for their controlled, well documented, and virtually identical genotype resulting from a sequence of at least 20 generations of inbreeding, and are typically referred to as distinct strains, rather than stocks or colonies, with their own codes denoting the outbred line from which they originated.

When designing an animal experiment, there are numerous factors that must be carefully considered about the research model to adequately control the environment for the purpose of transparent explanation of findings and facilitate reproducibility; these include sex, age, physiological status (pregnant or diseased), nutrition, cohabitation, genetic history, and other factors. It is also important to consider the applicability of the research, and so the majority of toxicologists have historically used, and continue to use, genetically heterogeneous outbred stocks of rats on the grounds that the it is most relevant to humans and their heterogeneous genetic make-up. However, this strategy decreases the degree of control that the researcher has in the laboratory setting, increases variability, and can reduce statistical power, as the exact genetic lineage of the animal is ultimately unknown, and any conclusions alluding to genetic variability are unfounded simply due to lack of specific characterization or description (Festing 2010).

Although outbred stocks are known to be variable in genotype, attempts have been made to characterize the phenotypic variability of both outbred and inbred rat stocks and strains in comparison to others, mainly to raise awareness that one strain or stock can potentially differ from another in respect to its response to any particular chemical or toxicant (Kacew & Festing 1999). Similarly here, we attempted to characterize the sex steroid hormone profiles of three popularly-used outbred stocks with respect to other major covariates such as age and sex. Information about the patterns in their sex hormones may facilitate the interpretation of historical studies that have used outbred lines. In this study, we gathered reported circulating peripheral concentrations of three steroid sex hormones—17-beta-estradiol (E2), progesterone (P4), and testosterone (T)—across the available literature that have utilized three common

outbred stocks (referred to hereon as strains for brevity), Sprague-Dawley, Wistar, and Long-Evans. We hypothesized that there will be recognizable strain variations in hormone concentrations, with sex-specific hormone patterns depending on age.

Methods

Article search

Pubmed queries started from the earliest date available date (1956) until February of 2015. Google Scholar searches of the same queries were investigated up to the first 500 results when sorted by Google's algorithmic determination of relevance due to the high volume of results. The two databases were searched based on combinations of the following keywords: estrogen, progesterone, testosterone, rat, plasma, serum, Sprague Dawley, Long Evans, and Wistar.

Then, articles were filtered using the specific inclusion criteria: (1) physiologically and anatomically normal; (2) untreated (naïve) baseline control animals; (3) vehicle control animals if the vehicle has been demonstrated to have minimal effect on the physiological processes of any organ system; (4) vehicle control animals if the vehicle volume did not exceed recommended volumes for rats (0.5 ml per 100 kg bw) (Turner, et al., 2011) or over 0.5% ethanol (Rachdaoui and Sakar 2013). We also excluded studies that: (1) used substances known to alter baseline hormone levels or general physiology, e.g., exogenous sex steroid hormones, soybean oil (rich in phytoestrogens); abnormal diet conditions, e.g., high caloric diets, starvation, high fat; high-dose radiation; carcinogenic substances; (2) hormone concentrations from animals post-surgery

(sham or experimental); (3) animals subjected to invasive procedures such as implantation or cannulation; (4) diseased; and (5) pregnant or pseudopregnant rats.

Data collected from each article

We recorded bibliographic information: article title, authors, journal, and publication date. We recorded summary statistics, e.g., means, medians, standard error (SE) or deviation (SD), 95% confidence interval, minimum and maximum levels. Methodological details collected included: number of animals used, tissue type (plasma or serum), and analytical technique (radioimmunoassay, ELISA, HPLC, etc.). We recorded any demographic data about the rats, e.g., sex, age (postnatal day and/or age group category), breed, weight, estrous cycle stage, time of day, and diet (e.g., soy-based or xenoestrogen-free).

Determination of statistical distributions

Prior to analysis, the distributions of estrogen, progesterone, and testosterone concentrations were determined using the published number of animals per group and summary statistics. The data were determined to have lognormal distributions based on one or more of the following criteria: (1) review publications that reported that biological data usually have a log-normal distribution; (2) the publication from which hormone data was extracted stated that the distribution(s) was log-normal; (3) the mean and median were not equal; (4) the mean was less than twice the SD, the CI included zero, or the minimum was negative; (5) we compared the fit of the data for individual animals (when reported) to normal and lognormal distributions. The arithmetic mean and SD or SE of hormone groups were used in the equation

described below to determine the parameters (estimated population mean and standard deviation, $\hat{\mu}$ and $\hat{\sigma}$, respectively) of the lognormal distribution. Using the program Lognorm4 (Strom & Stansbury, 2000), or after coding the relevant equations in Excel, Systat 13, or SAS 9.3, we determined the lognormal distribution using any two published values of the arithmetic standard deviation, mean, median, minimum, or maximum, or the arithmetic 95% CI.

Simulated dataset generation

The summary statistics for the lognormal distribution of a particular hormone, in a specific study, were used to create a random sample of the same size, log-mean, and log-SD reported in the study. When the size of the sample from the specific study for the particular hormone was sufficiently large (arbitrarily ≥20), the distribution of the random logarithmic values was checked for outliers and other departures from normality using probability plots. The underlying assumption for pooling data from different studies is that because animals used in a study is a random sample from the population of the selected strain, the summary statistics from all publications describe random samples from the same underlying, but unknown, lognormal distribution for each hormone. Therefore, the simulated logarithmic values compiled from all samples from all studies are expected to have a normal distribution. Custom-designed programs created in SAS 9.3 were used to carry out the simulations.

Data analysis

The set of all the random samples for each hormone separately was analyzed using the generalized linear mixed model procedure, GLIMMIX (SAS 9.4), to determine the extent to which

kit (radioimmunoassay, “RIA”; ELISA, “EIA”), tissue (serum, ”S”; plasma, “P”), strain (Long-Evans, “LE”; Sprague-Dawley, “SD”, Wistar, “W”), age (neonatal, pre-weaning, prepubertal, adult, aging), and sex (male or female) affected the mean level of each hormone. All variables in the model were fixed independent factors (i.e., were chosen by the original researcher and were specified in the inclusion criteria). A random variable, Paper ID, designated the random values as repeated measures (rats within study). Interactions retained in the model were age category within sex “AGE_CAT(SEX),” age category within strain “AGE_CAT(STRAIN),” strain by sex “STRAIN*SEX,” and age category within sex by strain “AGE_CAT(SEX*STRAIN).” Age category was a nested factor, rather than crossed, because some age categories were not found during literature review for both sexes and all strains. Four F test methods (Tukey’s HSD, Hochberg’s step-up Bonferroni, Holm’s step-down Bonferroni, and False Discovery Rate) to adjust p-values for multiple comparisons were compared. Adjusted P-values for all effects are in the tables and p-values are in the text by adjustment method. Residuals from the ANOVA were checked for normality using probability and quartile-quartile (Q-Q) plots, and histograms.

Results

Kit and Tissue Effect

Main effects for kit were non-significant for estradiol, progesterone, and testosterone (Tables 1, 2). Main effects and pairwise comparisons for tissue were significant for progesterone and testosterone, but not for estradiol (Tables 1, 3).

Sex Effect

Pairwise comparisons were not estimable for any interaction that included age category due to multiple missing combinations (Table 15, Figure 22). For the same reason, sex differences by strain averaged over all age categories were not estimable for all three hormones.

Estradiol:

The main effect of sex for estradiol was not significant ($p = 0.055$); however, the interactions of sex (within age category, averaged over all strains) and sex (by strain, within age-category) were significant ($p < 0.0001$, $p = 0.0084$, respectively) (Table 4). The interaction of sex (by strain, averaged over all age categories) was not significant ($p = 0.11$) (Table 4).

Pairwise differences of estradiol between males and females within the prepubertal age category (averaged over all strains) was significant, with males having higher estradiol than females (Holm, Hoch, FDR $p = 0.027$) (Table 5, Figure 1). Comparisons of sex for the neonatal, preweaning, and aging categories could not be estimated.

Significant pairwise differences in estradiol were found between males and females (within age category, by strain), in which adult females had higher estradiol than males for adult Long-Evans (Holm $p = 0.014$; Hoch $p = 0.014$; FDR $p = 0.013$), and vice versa for neonatal Wistar (Holm, Hoch $p = 0.023$; FDR $p = 0.013$) and prepubertal Sprague-Dawley (FDR $p = 0.038$) (Table 6, Figure 4). The inconsistency between the significant main interaction of sex and age category and the non-significance of pairwise comparisons within the interaction is due to the adjustment of p-values for the pairwise comparisons. Comparisons of sex for the Long-Evans neonatal and preweaning age categories could not be estimated.

Progesterone:

For progesterone, the main effect for sex was not statistically significant ($p = 0.17$) but the interaction of sex within age category averaged over all strains, was significant ($p = 0.0007$) (Table 4). The interaction of sex by strain averaged over all age categories, and the interaction of sex by strain within age-category, were not significant ($p = 0.11$, $p = 0.35$, respectively) (Table 4).

For pairwise comparisons, progesterone concentrations differed significantly between sexes within each age category averaged over all strains, with female adults higher than male adults (Tukey $p = 0.027$; Holm, Hoch, FDR $p = 0.002$) (Table 5, Figure 2). Comparisons of sex for the neonatal, preweaning, and aging categories could not be estimated.

There were significant pairwise differences between sexes within each age category, by strain, in which female adult Sprague-Dawley had higher estradiol levels than male adult Sprague-Dawley (Holm, Hoch, FDR $p = 0.008$) (Table 6, Figure 5). Comparisons of sex for the Long-Evans neonatal and preweaning age categories could not be estimated due to insufficient data.

Testosterone:

The main effect of sex for testosterone was significant ($p < 0.0001$), as well as interactions of sex within age category averaged over all strains ($p = 0.033$), and sex by strain within age category ($p < 0.0001$), but not for the interaction of sex by strain averaged over all age categories ($p = 0.31$) (Table 4).

Pairwise differences of testosterone between males and females within age category, averaged over all strains, were significant for adults, with males greater than females (Tukey, Holm, Hoch, FDR $p < 0.0001$) (Table 5, Figure 3). Comparisons within all other age categories could be estimated.

All pairwise differences of testosterone between males and females within age category, by strain, were significant, with males higher than females in all cases (all Hoch, FDR $p < 0.04$) (Table 6, Figure 6). Comparisons of sex for Long-Evans could not be estimated for the neonatal and preweaning age categories, and Wistar could only be estimated for adults.

Age Effect

Pairwise comparisons were not estimable for any interaction that included the "aging" category due to insufficient numbers of values.

Estradiol:

The main effect of age category for estradiol was significant ($p < 0.0001$), as were the interactions of sex within age category, averaged over all strains ($p < 0.0001$), and strain within age category, averaged over both sexes ($p = 0.0084$) (Table 4). The interaction of sex by strain, within age category, was not significant ($p = 0.23$) (Table 4).

Pairwise differences of estradiol between age categories averaged over both sexes and all strains were all significant, but could only be estimated for the comparison of neonatal-preweaning ($p = 0.0007$) and prepubertal-adult ($p < 0.0001$) (Table 7). The preweaning group

had higher estradiol levels than the neonatal group, and the prepubertal group had higher estradiol levels than the adult group.

Pairwise comparisons of age categories for females averaged over all strains reported the preweaning group had significantly higher estradiol than the neonatal age group (Tukey $p < 0.0001$; Holm $p = 0.0004$; Hoch $p = 0.0002$; FDR $p = 0.00013$), and the prepubertal group had significantly higher estradiol than the adult age group (Tukey $p < 0.0001$; Holm $p = 0.0004$; Hoch $p = 0.0002$; FDR $p = 0.00013$) (Table 8, Figure 7). For males, the prepubertal group also had significantly higher estradiol than the adult group (Tukey $p < 0.0001$; Holm $p = 0.0004$; Hoch $p = 0.0002$; FDR $p = 0.00013$). The only other comparison that could be estimated was between male neonatal and preweaning groups, and this comparison was not significant (Tukey $p = 0.69$; Holm, Hoch, FDR $p = 0.0901$) (Table 8).

Significant pairwise differences between age categories within sex by strain were found between the majority of pairings of age categories, for at least one strain per pairing, except for the comparison of male neonatal and prepubertal age groups (SD: all adjusted $p > 0.5$; Wistar: all adjusted $p > 0.3$) and male preweaning and prepubertal age groups (SD: all adjusted $p > 0.8$; Wistar: all adjusted $p > 0.2$) (Table 10, Figure 13). Except for the comparisons of prepubertal-adult, and prepubertal-aging female age categories, comparisons of age categories for the Long-Evans strain could not be estimated. Overall, there were significantly higher estradiol levels during prepuberty than adulthood for all strains and in both sexes, except for female Wistars. Although the comparison of neonatal and preweaning age groups could not be estimated for Long-Evans, there were significantly higher estradiol levels during the preweaning versus neonatal period for all strains and in both sexes, except for male Wistar rats.

Progesterone:

The main effect of age category for progesterone was significant ($p < 0.0001$), as were the interactions of sex within age category averaged over all strains ($p = 0.0007$), and strain within age category averaged over both sexes ($p = 0.0002$) (Table 4). The interaction of sex by strain within age category was not significant ($p = 0.35$) (Table 4).

Pairwise differences of progesterone between age categories averaged over both sexes and all strains were significant for the comparison of neonatal-preweaning ($p = 0.041$), but not for prepubertal-adult ($p = 0.73$) (Table 7). All other age comparisons could not be estimated. The preweaning group had higher estradiol than the neonatal group.

There were no significant pairwise differences between age categories within sex averaged over all strains (all $p > 0.2$) (Table 8, Figure 8). Only comparisons of neonatal-preweaning, prepubertal-adult, prepubertal-aging, and adult-aging could be estimated for females, and only neonatal-preweaning and prepubertal-adult could be estimated for males.

Significant pairwise differences between age categories within sex by strain were found between the majority of pairings of age categories for females, for at least one strain per pairing (all $p < 0.02$) (Table 10, Figure 14). Overall, progesterone significantly increased with age until adulthood. Except for the pairings involving any two combinations of the prepubertal, adult, or aging categories, comparisons of age categories for the Long-Evans strain could not be estimated for females. Pairwise differences between age categories within sex by strain were found with the neonatal group having significantly lower progesterone than all other age categories, except for aging (which could not be estimated) for Sprague-Dawley only in males (all $p < 0.04$) (Table

10, Figure 14). Long-Evans age comparisons could only be estimated for the comparison of prepubertal-adult in males.

Testosterone:

The main effect of age category for testosterone was significant ($p < 0.0001$), as were the all interactions involving age category, including sex within age category averaged over all strains) ($p = 0.033$), strain within age category averaged over both sexes ($p < 0.0001$), and sex by strain within age category ($p < 0.0001$) (Table 4).

None of the pairwise differences of testosterone between age categories averaged over both sexes and all strains, or pairwise differences between age categories by sex averaged over all strains, could be estimated (Table 7, Table 8).

For females, significant pairwise differences between age categories by sex by strain, were found between the majority of pairings of age categories, for at least one strain per pairing (all $p < 0.006$) (Table 10, Figure 15), except for neonatal-adult ($p = 0.070$). Except for the prepubertal-aging and adult-aging, comparisons of age categories for the Long-Evans strain could not be estimated for females. Wistar comparisons could only be estimated for preweaning-prepubertal, preweaning-adult, and prepubertal-adult for females.

For males, significant pairwise differences between age categories by sex by strain, were found between neonatal-prepubertal (Long-Evans) (all $p < 0.003$), neonatal-prepubertal (Long-Evans and Sprague-Dawley) (all $p < 0.003$), and prepubertal-adult (Long-Evans and Sprague-Dawley) (all $p < 0.003$) (Table 10, Figure 15). Comparisons involving the aging category could not be estimated. Comparisons of within the Wistar strain could not be estimated for any pairing of

age categories, and within the Long-Evans strain could only be estimated for neonatal-prepubertal, neonatal-adult, and prepubertal-adult for males.

Strain Effect

Estradiol:

The main effect of strain for estradiol was not significant ($p = 0.097$) (Table 4). The interaction of strain by sex averaged over all age categories, and the interaction of sex by strain within age category, were not significant ($p = 0.11$, $p = 0.23$, respectively). However, the interaction of strain within age category averaged over both sexes was significant ($p = 0.0084$).

Pairwise differences of estradiol between strains averaged over both sexes and all age categories could only be estimated for the comparison of Sprague-Dawley with Wistar, which was not significant ($p = 0.64$) (Table 11). Pairwise differences of estradiol between strains by sex averaged over all age categories could only be estimated for the comparison of the Sprague-Dawley with Wistar strains for males and females. Only the comparison of female Sprague-Dawley with Wistar rats was significant (Tukey: $p = 0.088$; Holm, Hoch, and FDR: $p = 0.038$), with estradiol levels higher in the Sprague-Dawley than Wistar strain (Table 12).

For females, pairwise comparisons of strain within age category by sex were significant between the Sprague-Dawley and Wistar strains, with the Sprague-Dawley strain having higher estradiol levels than the Wistar strain in the neonatal and preweaning periods (all adjusted $p < 0.05$) (Table 14, Figure 19). During adulthood, the Sprague-Dawley strain also had significantly higher estradiol than Long-Evans strain (only for the False Discovery Rate, $p = 0.025$). The Wistar

strain also had significantly higher estradiol levels than the Long-Evans strain (all adjusted $p < 0.04$).

For males, pairwise comparisons of strain within age category by sex were similar to females in that both the Sprague-Dawley and Wistar strains estradiol levels were significantly higher than in the Long-Evans strain (only for the False Discovery Rate, SD-LE: $p = 0.025$, W-LE: $p = 0.040$). For both males and females, pairwise comparisons were not estimable for any pairing that involved the aging category. Strain comparisons within the neonatal and preweaning age categories could only be estimated for the comparison of Sprague-Dawley with Wistar strains.

Progesterone:

The main effect of strain for progesterone was not significant ($p = 0.99$) (Table 4). The interaction of strain by sex averaged over all age categories and the interaction of sex by strain within age category were not significant ($p = 0.13$, $p = 0.35$, respectively). However, the interaction of strain within age category averaged over both sexes was significant ($p = 0.0002$).

Pairwise differences of progesterone between strains averaged over both sexes and all age categories, and pairwise differences between strains by sex averaged over all age categories, could not be estimated (Table 11, Table 12).

No significant pairwise differences were found between strains within age category by sex (Table 14, Figure 20). Comparisons within the neonatal and preweaning age categories could only be estimated for the comparison of Sprague-Dawley and Wistar strains for males and females. Comparisons within the aging group could only be estimated for females.

Testosterone:

The main effect of strain for testosterone was not significant ($p = 0.81$) (Table 4). The interaction of strain by sex averaged over all age categories was not significant ($p = 0.32$), However, the interaction of strain within age category averaged over both sexes, and the interaction of strain within age category by sex, were significant (both $p < 0.0001$).

Pairwise differences of testosterone between strains averaged over both sexes and all age categories, and pairwise differences between strains by sex averaged over all age categories, could not be estimated (Table 11, Table 12).

For females, pairwise comparisons of strain within age category by sex were significant between the Sprague-Dawley and Wistar strains, with the Sprague-Dawley strain having higher testosterone levels than the Wistar strain during the prepubertal period (only for the False Discovery Rate, $p = 0.038$) (Table 14, Figure 21). Significant differences were found between the Long-Evans and Sprague-Dawley strains, with Sprague-Dawley having higher testosterone levels during prepuberty (only for the False Discovery Rate, $p = 0.044$), and with Long-Evans having higher testosterone during adulthood (only for the False Discovery Rate, $p = 0.032$). Comparisons within the preweaning age category could only be estimated for the comparison of the Sprague-Dawley with Wistar strains. Pairwise comparisons were not estimable for the neonatal or aging categories.

For males, pairwise comparisons of strain (within age category, by sex) were significant between the Sprague-Dawley and Wistar strains, with Wistar rats having higher testosterone levels than Sprague-Dawley males during the adult period (all adjusted $p < 0.02$) (Table 14, Figure 21). Significant differences were found between the Long-Evans and Sprague-Dawley

strains, with the Sprague-Dawley strain having the higher testosterone levels during the neonatal period (only for the False Discovery Rate, $p = 0.032$). The Long-Evans strain had the higher testosterone levels during prepuberty (only for the False Discovery Rate, $p = 0.038$). The Wistar strain had significantly higher testosterone levels than the Long-Evans strain during adulthood (only for the False Discovery Rate, $p = 0.042$). Pairwise comparisons within the neonatal and prepubertal age categories could only be estimated for the comparison of the Long-Evans with Sprague-Dawley strains, and no comparisons could be estimated for the preweaning or aging categories.

Discussion

In the study of reproductive toxicology, one of the greatest difficulties is establishing reproducible results for a chemical of interest. The controversy over the contradictory results obtained by different laboratory groups can potentially be traced to differences in laboratory technique, experimental subjects, and other aspects of experimental design. In particular, the chemical bisphenol-A has established itself as one of these controversial chemicals in recent years. Kwon, et al., (2000), who conducted a BPA study on development and reproductive function, posited an interesting point by stating that one of the factors that may have affected their results could be the choice in strain of their rodent model, and "due to the insensitivity of Sprague-Dawley rats to endocrine-mediated toxicity by endocrine modulators." However, they further elaborated that this potential for strain-related differences in susceptibility is under-characterized, and that genetically mediated mechanisms of endocrine toxicity have yet to be clearly defined. In addition to acknowledging interspecies differences in the study of endocrine disruption, laboratories must also consider the susceptibility of a particular genotype within a

species. While much of the functional activity of sex hormones has been studied and established, hormonal sensitivity and the factors governing differences within a population have received less attention (Spearow, et al., 1999). To characterize genotypic susceptibility profiles, one can begin by describing the traits attributable to a particular strain in comparison to others. In this instance, we investigated strain differences in steroidal sex hormone levels with respect to sex and age.

As expected, there were significant sex and age differences in hormone levels. Males had higher levels of testosterone at all ages and in several strains (Table 6). Sex differences in estradiol were less prominent, though adult females had higher estradiol than males within at least one strain, while males had higher estradiol within the age groups prior to puberty. There was little difference between sexes in progesterone, except within the adult age category when females began to show higher levels than males. Age differences in all hormones were present: estradiol appeared to increase after the neonatal period and then dropped after puberty in females (Table 8). This seems contradictory to what is known about the role of rising estradiol that occurs during puberty in females, although the influence of estrous cycle may have played a part in this discovery, as estrous cycle stage of adult females was captured during data compilation (when given) but was not included in the model in this preliminary analysis (to accommodate missing data). Future evaluation of the data could and should take into consideration the particular stage within the estrous cycle from which the measurements were taken. Progesterone and testosterone both seemed to increase steadily with age for both sexes, with the exception of a dip in female testosterone occurring between the preweaning and prepubertal periods (Table 10).

Our analysis showed no differences between the popular outbred strains of Long-Evans, Wistar, or Sprague-Dawley in the levels of circulating progesterone at any age (Table 14). In contrast, circulating levels of estradiol in the Long-Evans strain appeared to be lower than both Sprague-Dawley and Wistar strains for both male and female adult animals (Table 14). This is consistent with the findings of O'Connor, et al., (1999), who found that serum estradiol was significantly greater for Sprague-Dawley rats compared to Long-Evans rats. Female Wistar rats also had less estradiol than young (neonatal and preweaning) Sprague-Dawley rats. Accordingly—although the study was conducted in adult animals—female Wistar rats were more sensitive to the uterine proliferating effects of ethinyl estradiol than Sprague-Dawley rats (Diel, et al., 2001), which may be explained by the larger pool of estradiol available to buffer the effects of exogenous estrogenic compounds in Sprague-Dawley rats.

Interestingly, Sprague-Dawley rats of both sexes had higher testosterone levels than Long-Evans rats early on (female prepubertal rats, and male neonatal rats), but this strain difference flipped as the animals aged: when females reached adulthood, and males reached pre-puberty, Long-Evans rats had higher amounts of testosterone than Sprague-Dawley rats (Table 14). Anogenital distance at PND 2 was found to be slightly less (~ 1mm) for male Long-Evans rats compared to Sprague-Dawley rats, which supports our finding that these animals had less testosterone at young ages because shorter anogenital distance is an indicator of feminization and lower androgen levels (You, et al., 1998). Furthermore, the Sprague-Dawley rats in this study were less susceptible than the Long-Evans rats to the anogenital-distance- and seminal-vesicle-weight-reducing effects of androgen antagonist, *p,p'*-DDE. The strain difference also depended on the sex of the animal, as male Wistar rats had higher testosterone than both

Sprague-Dawley and Long-Evans strains, but female Wistar rats had lower testosterone levels than the Sprague-Dawley strain. Wilkinson, et al., (2000), reported that male Wistar rats had significantly larger testes relative to body weight than Sprague-Dawley rats, consistent with our finding that these animals also had higher testosterone levels, although the Sprague-Dawley rats had larger seminal vesicles, an organ in which development, size, and maintenance are dependent on androgens.

Conclusions

These analyses indicate that there are apparent differences in hormone levels between the commonly utilized Sprague-Dawley, Wistar, and Long-Evans strains, and that these strain differences are also dependent on the animal's sex and age. What mechanisms underlie the cause of these strain differences should be a topic of further genetic evaluation. Hypothesized mechanisms have already been proposed such as differences in the speed and efficiency of pharmacokinetic metabolism (You, et al., 1998; O'Connor, et al., 1999; Spearow, et al., 1999), alternate activation of hormonally responsive genes (Long, et al., 2000), and steroidogenesis and/or gonadotropin secretion rates (Spearow, et al., 1999). However, these proposed mechanisms are likely to be dependent on tissue type.

Significant strain-specific sensitivity to sex-hormone agonists and antagonists and other endocrine-disrupting chemicals have already been established with regard to other reproductive parameters in addition to sex hormone levels (Howdeshell, et al., 2008; Bailey & Nephew, et al., 2002; Pandey, et al., 2005; Long, et al., 2000; Wilkinson, et al., 2000; Steinmetz, et al., 1997; You, et al., 1998; Tyl, et al., 2002; O'Connor, et al., 1999). Thus, care must be taken in toxicological

assessments to consider the traits of any particular strain before comparison with other rodent studies, and with human populations. Future studies providing information on strain differences in various reproductive measurements combined with knowledge of strain-specific differences in susceptibility to endocrine-mediated toxicity will enhance our understanding of the mechanisms by which a toxicant affects an animal's physiology.

It is appropriate to conclude with a word of caution: some of the "not significant" statistical differences are due to small sample sizes for some combinations of the independent variables. The statistical analysis reports these as "not estimable," which is not the same as "not significant" and "inestimable given a larger sample." Statements of "not significant" deriving from sample characteristics must be distinguished from "not-significant" due to differences in hormone levels that are truly small. The overall caveat is that "not significant," as for "significant," applies to the statistical properties of the data used in the analysis, which may or may not reflect the biological consequences of such differences, overall (for the "sample") and for the individual animals comprising the sample.

Tables

Table 1. Kit and Tissue Main Effects: Type III Tests of Fixed Effects

	Effect	Num DF	Den DF	F Value	Pr > F
E2	KIT	1	3139	0.02	0.87
	TISSUE	1	3139	0.00	0.98
P4	KIT	1	3326	0.20	0.65
	TISSUE	1	3326	20.2	*<0.0001
T	KIT	1	2982	1.11	0.29
	TISSUE	1	2982	12.5	*0.0004

Separate analyses run by hormone.
*statistically significant according to critical value, alpha = 0.05.

Table 2. Differences of Kit LSMs (EIA vs. RIA)

	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P (Tukey)
E2	-0.0383	0.249	3139	-0.15	0.88	0.87
P4	0.169	0.378	3326	0.45	0.66	0.65
T	0.126	0.120	2982	1.06	0.29	0.29

Separate analyses run by hormone.
*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 3. Differences of Tissue LSMs (Plasma vs. Serum)

	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P (Tukey)	
E2	-0.00338	0.252	3139	-0.01	0.99	0.98	
P4	-0.645	0.144	3326	-4.49	<.0001	*<0.0001	S > P
T	0.466	0.132	2982	3.53	0.0004	*0.0004	P > S

Separate analyses run by hormone.
*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 4. Sex, Strain, and Age Main Effects and Interactions: Type III Tests of Fixed Effects

	Effect	Num DF	Den DF	F Value	Pr > F
E2	SEX	1	3139	3.69	0.055
	STRAIN	2	3139	2.34	0.097
	AGE_CAT	4	3139	26.3	*<.0001
	STRAIN*SEX	2	3139	2.19	0.11
	AGE_CAT(SEX)	3	3139	7.13	*<0.0001
	AGE_CAT(STRAIN)	4	3139	3.43	*0.0084
	AGE_CAT(STRAIN*SEX)	4	3139	1.41	0.23
P4	SEX	1	3326	1.92	0.17
	STRAIN	2	3326	0.01	0.99
	AGE_CAT	4	3326	17.9	*<0.0001
	STRAIN*SEX	2	3326	2.05	0.13
	AGE_CAT(SEX)	3	3326	5.72	*0.0007
	AGE_CAT(STRAIN)	6	3326	4.43	*0.0002
	AGE_CAT(STRAIN*SEX)	4	3326	1.12	0.35
T	SEX	1	2982	65.8	*<0.0001
	STRAIN	2	2982	0.21	0.81
	AGE_CAT	4	2982	68.3	*<0.0001
	STRAIN*SEX	2	2982	1.16	0.31
	AGE_CAT(SEX)	3	2982	2.92	*0.033
	AGE_CAT(STRAIN)	4	2982	20.0	*<0.0001
	AGE_CAT(STRAIN*SEX)	1	2982	35.5	*<0.0001

Separate analyses run by hormone. Analyses were run simultaneously with effects listed in Table 1, according to respective hormones.
*statistically significant according to critical value, alpha = 0.05.

Table 5. Sex Effect: Differences of AGE_CAT(SEX) LSMs (Female vs. Male)

	Age	Estimate	SE	DF	t Value	Pr > |t|	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
E2	1. neonatal	Non-est	.	.	.	.	.				
	2. preweaning	Non-est	.	.	.	.	.				
	3. prepubertal	-0.723	0.293	3139	-2.47	0.0137	0.21	*0.027	*0.027	*0.027	M>F
	4. adult	0.0942	0.249	3139	0.38	0.705	0.99	0.71	0.71	0.71	
P4	1. neonatal	Non-est	.	.	.	.	.				
	2. preweaning	Non-est	.	.	.	.	.				
	3. prepubertal	0.170	0.317	3326	0.54	0.591	0.99	0.59	0.59	0.59	
	4. adult	0.863	0.262	3326	3.30	0.0010	*0.027	*0.002	*0.002	*0.002	F>M
T	1. neonatal	Non-est	.	.	.	.	.				
	2. preweaning	Non-est	.	.	.	.	.				
	3. prepubertal	Non-est	.	.	.	.	.				
	4. adult	-1.94	0.212	2982	-9.17	<0.0001	*<0.0001	*<0.0001	*<0.0001	*<0.0001	M>F

Separate analyses run by hormone.
*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 6. Sex Effect: Differences of AGE_CAT(STRAIN*SEX) LSMs (Female vs. Male)

	Age	Strain	Estimate	SE	DF	t Value	Pr > |t| (Raw)	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
E2	1. neonatal	SD	-0.589	0.992	3139	-0.59	0.55	1	1	0.92	0.69	
		Wistar	-1.92	0.634	3139	-3.03	0.0025	0.23	*0.023	*0.023	*0.013	M>F
	2. preweaning	SD	-0.764	1.012	3139	-0.75	0.45	1	1	0.92	0.69	
		Wistar	-1.44	0.627	3139	-2.3	0.022	0.75	0.151	0.15	0.054	
	3. prepubertal	LE	-0.0243	0.232	3139	-0.1	0.92	1	1	0.92	0.92	
		SD	-1.35	0.533	3139	-2.53	0.011	0.58	0.090	0.090	*0.038	M>F
		Wistar	-0.796	0.624	3139	-1.28	0.20	0.99	1	0.92	0.41	
	4. adult	LE	0.511	0.160	3139	3.19	0.0014	0.16	*0.014	*0.014	*0.013	F>M
		SD	-0.326	0.489	3139	-0.67	0.51	1	1	0.92	0.69	
		Wistar	0.0972	0.497	3139	0.2	0.85	1	1	0.92	0.92	
P4	1. neonatal	SD	0.972	0.454	3326	2.14	0.033	0.88	0.293	0.29	0.14	
		Wistar	-0.148	0.700	3326	-0.21	0.83	1	1	0.83	0.83	
	2. preweaning	SD	0.609	0.451	3326	1.35	0.18	0.99	1	0.83	0.36	
		Wistar	-0.232	0.551	3326	-0.42	0.67	1	1	0.83	0.83	
	3. prepubertal	LE	0.233	0.275	3326	0.8f5	0.40	1	1	0.83	0.66	
		SD	0.434	0.607	3326	0.72	0.47	1	1	0.83	0.68	
		Wistar	-0.156	0.547	3326	-0.28	0.78	1	1	0.83	0.83	
	4. adult	LE	0.368	0.180	3326	2.05	0.041	0.92	0.327	0.33	0.14	
		SD	1.63	0.487	3326	3.34	0.0008	0.11	*0.008	*0.008	*0.008	F>M
		Wistar	0.595	0.438	3326	1.36	0.17	0.99	1	0.83	0.36	
T	1. neonatal	SD	-1.52	0.385	2982	-3.95	<0.0001	*0.01	*0.001	*<0.0001	*<0.0001	M>F
	2. preweaning	SD	-0.891	0.426	2982	-2.09	0.037	0.82	0.067	*0.037	*0.037	M>F
	3. prepubertal	LE	-2.81	0.364	2982	-7.72	<0.0001	*<0.0001	*0.001	*<0.0001	*<0.0001	M>F
		SD	-0.892	0.420	2982	-2.12	0.033	0.80	0.067	*0.037	*0.037	M>F
	4. adult	LE	-1.33	0.134	2982	-9.98	<0.0001	*<0.0001	*<0.0001	*<0.0001	*<0.0001	M>F
		SD	-2.16	0.357	2982	-6.05	<0.0001	*<0.0001	*<0.0001	*<0.0001	*<0.0001	M>F
		Wistar	-2.32	0.500	2982	-4.65	<0.0001	*0.0005	*<0.0001	*<0.0001	*<0.0001	M>F

Separate analyses run by hormone.
*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 7. Age Effect: Differences of AGE_CAT LSMs

	AGE_CAT	AGE_CAT	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P	
E2	1. neonatal	2. preweaning	-0.4259	0.1109	3139	-3.84	0.0001	*0.0007	2>1
		3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	2. preweaning	3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	3. prepubertal	4. adult	1.0837	0.1145	3139	9.47	<.0001	*<.0001	3>4
		5. aging	Non-est	.	.	.	.	.	
	4. adult	5. aging	Non-est	.	.	.	.	.	
P4	1. neonatal	2. preweaning	-0.4825	0.1824	3326	-2.65	0.0082	*0.0409	2>1
		3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	2. preweaning	3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	3. prepubertal	4. adult	-0.4112	0.3959	3326	-1.04	0.2991	0.7268	
		5. aging	Non-est	.	.	.	.	.	
	4. adult	5. aging	Non-est	.	.	.	.	.	
T	1. neonatal	2. preweaning	Non-est	.	.	.	.	.	
		3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	2. preweaning	3. prepubertal	Non-est	.	.	.	.	.	
		4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	3. prepubertal	4. adult	Non-est	.	.	.	.	.	
		5. aging	Non-est	.	.	.	.	.	
	4. adult	5. aging	Non-est	.	.	.	.	.	

Separate analyses run by hormone.

*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 8. Age Effect: Differences of AGE_CAT(SEX) LSMs

	SEX	AGE_CAT	AGE_CAT	Estimate	SE	DF	t Value	Pr > |t|	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
E2	F	1. neonatal	2. preweaning	-0.5015	0.08079	3139	-6.21	<.0001	*<.0001	*0.0004	*0.0002	*0.00013	2>1
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
			5. aging	Non-est	.	.	.	.	.				
		2. preweaning	3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
			5. aging	Non-est	.	.	.	.	.				
		3. prepubertal	4. adult	0.6750	0.1140	3139	5.92	<.0001	*<.0001	*0.0004	*0.0002	*0.00013	3>4
			5. aging	Non-est	.	.	.	.	.				
	M	1. neonatal	2. preweaning	-0.3504	0.2066	3139	-1.70	0.0901	0.6902	0.0901	0.0901	0.0901	
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		2. preweaning	3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		3. prepubertal	4. adult	1.4925	0.1734	3139	8.61	<.0001	*<.0001	*0.0004	*0.0002	*0.00013	3>4
P4	F	1. neonatal	2. preweaning	-0.3709	0.1471	3326	-2.52	0.0117	0.2219	0.0702	0.0702	0.0558	
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
			5. aging	Non-est	.	.	.	.	.				
		2. preweaning	3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
			5. aging	Non-est	.	.	.	.	.				
		3. prepubertal	4. adult	-0.7577	0.3927	3326	-1.93	0.0538	0.5933	0.2152	0.2152	0.1076	
			5. aging	-0.5050	0.4068	3326	-1.24	0.2146	0.9473	0.4292	0.4292	0.25752	
		4. adult	5. aging	0.2527	0.1073	3326	2.36	0.0186	0.3095	0.093	0.093	0.0558	
	M	1. neonatal	2. preweaning	-0.5942	0.3339	3326	-1.78	0.0752	0.6959	0.2256	0.2256	0.1128	
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		2. preweaning	3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		3. prepubertal	4. adult	-0.06463	0.4266	3326	-0.15	0.8796	1	0.8796	0.8796	0.8796	
T	F	1. neonatal	2. preweaning	Non-est	.	.	.	.	.				
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
			5. aging	Non-est	.	.	.	.	.				
	M	1. neonatal	2. preweaning	Non-est	.	.	.	.	.				
			3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		2. preweaning	3. prepubertal	Non-est	.	.	.	.	.				
			4. adult	Non-est	.	.	.	.	.				
		3. prepubertal	4. adult	Non-est	.	.	.	.	.				

Separate analyses run by hormone.

*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 9. Age Effect: Differences of AGE_CAT(STRAIN) LSMs

	Age	Age	Strain	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
E2	1. neonatal	2. preweaning	SD	-0.5312	0.1197	3139	-4.44	<.0001	*0.0004
			W	-0.3207	0.1868	3139	-1.72	0.0861	0.7862
		3. prepubertal	SD	-0.4436	0.5506	3139	-0.81	0.4205	0.9985
			W	-0.1571	0.1838	3139	-0.85	0.3929	0.9976
		4. adult	SD	0.5910	0.5421	3139	1.09	0.2757	0.9857
			W	0.3775	0.2118	3139	1.78	0.0748	0.7465
	2. preweaning	3. prepubertal	SD	0.08765	0.5590	3139	0.16	0.8754	1
			W	0.1635	0.1783	3139	0.92	0.3591	0.9960
		4. adult	SD	1.1223	0.5510	3139	2.04	0.0418	0.5729
			W	0.6981	0.2066	3139	3.38	0.0007	*0.0255
	3. prepubertal	4. adult	LE	1.6820	0.2484	3139	6.77	<.0001	*<.0001
			SD	1.0346	0.1200	3139	8.62	<.0001	*<.0001
			Wistar	0.5346	0.2038	3139	2.62	0.0087	0.2068
		5. aging	LE	Non-est	.	.	.	.	.
	4. adult	5. aging	LE	Non-est	.	.	.	.	.
P4	1. neonatal	2. preweaning	SD	-0.5525	0.1574	3326	-3.51	0.0005	*0.0165
			Wistar	-0.4125	0.3286	3326	-1.26	0.2094	0.9630
		3. prepubertal	SD	-1.2679	0.2784	3326	-4.55	<.0001	*0.0002
			Wistar	-0.6311	0.3050	3326	-2.07	0.0386	0.5493
		4. adult	SD	-1.3353	0.2170	3326	-6.15	<.0001	*<.0001
			Wistar	-1.4331	0.2844	3326	-5.04	<.0001	*<.0001
		5. aging	SD	Non-est	.	.	.	.	.
			Wistar	Non-est	.	.	.	.	.
	2. preweaning	3. prepubertal	SD	-0.7154	0.2514	3326	-2.85	0.0045	0.1219
			Wistar	-0.2186	0.2428	3326	-0.90	0.3679	0.9965
		4. adult	SD	-0.7828	0.1835	3326	-4.27	<.0001	*0.0009
			Wistar	-1.0206	0.1962	3326	-5.20	<.0001	*<.0001
		5. aging	SD	Non-est	.	.	.	.	.
			Wistar	Non-est	.	.	.	.	.
	3. prepubertal	4. adult	LE	-0.3641	1.1554	3326	-0.32	0.7527	1
			SD	-0.06740	0.2013	3326	-0.33	0.7377	1
			Wistar	-0.8020	0.1872	3326	-4.28	<.0001	*0.0008
		5. aging	LE	Non-est	.	.	.	.	.
			SD	Non-est	.	.	.	.	.
			Wistar	Non-est	.	.	.	.	.
	4. adult	5. aging	LE	Non-est	.	.	.	.	.
			SD	Non-est	.	.	.	.	.
			Wistar	Non-est	.	.	.	.	.
T	1. neonatal	2. preweaning	SD	-0.8610	0.1091	2982	-7.89	<.0001	*<.0001
		3. prepubertal	LE	Non-est	.	.	.	.	.
			SD	-0.07693	0.1399	2982	-0.55	0.5826	0.9981
		4. adult	LE	Non-est	.	.	.	.	.
			SD	-0.6139	0.09694	2982	-6.33	<.0001	*<.0001
		5. aging	LE	Non-est	.	.	.	.	.
	2. preweaning	3. prepubertal	SD	0.7841	0.1651	2982	4.75	<.0001	*<.0001
			Wistar	Non-est	.	.	.	.	.
		4. adult	SD	0.2471	0.1329	2982	1.86	0.0630	0.5073
			Wistar	Non-est	.	.	.	.	.
	3. prepubertal	4. adult	LE	-1.3169	0.1937	2982	-6.80	<.0001	*<.0001
			SD	-0.5370	0.1253	2982	-4.29	<.0001	*0.0004
			Wistar	Non-est	.	.	.	.	.
		5. aging	LE	Non-est	.	.	.	.	.
	4. adult	5. aging	LE	Non-est	.	.	.	.	.

Separate analyses run by hormone.

*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 10. Age Effect: Differences of AGE_CAT(STRAIN*SEX) LSMs

	SEX	AGE_CAT	AGE_CAT	STRAIN	Estimate	SE	DF	t Value	Pr > |t|	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
E2	F	1. neonat	2. prewean	SD	-0.444	0.102	3139	-4.34	<.0001	*0.0027	*0.0028	*0.0018	*0.0003	2>1
				Wistar	-0.560	0.125	3139	-4.47	<.0001	*0.0015	*0.0028	*0.0018	*0.0003	2>1
			3. prepub	SD	-0.0630	0.0910	3139	-0.69	0.49	1	1	0.85	0.58	
				Wistar	-0.719	0.131	3139	-5.5	<.0001	*<.0001	*0.0028	*0.0018	*0.0003	3>1
			4. adult	SD	0.459	0.105	3139	4.36	<.0001	*0.0024	*0.0028	*0.0018	*0.0003	1>4
				Wistar	-0.631	0.118	3139	-5.36	<.0001	*<.0001	*0.0028	*0.0018	*0.0003	4>1
		2. prewean	3. prepub	SD	0.381	0.104	3139	3.67	0.0002	*0.036	*0.0034	*0.0032	*0.0004	2>3
				Wistar	-0.160	0.128	3139	-1.25	0.21	1	1	0.85	0.33	
			4. adult	SD	0.903	0.125	3139	7.26	<.0001	*<.0001	*0.0028	*0.0018	*0.0003	2>4
				Wistar	-0.0717	0.112	3139	-0.64	0.52	1	1	0.85	0.58	
		3. prepub	4. adult	LE	1.41	0.301	3139	4.7	<.0001	*0.0005	*0.0028	*0.0018	*0.0003	3>4
				SD	0.522	0.109	3139	4.77	<.0001	*0.0004	*0.0028	*0.0018	*0.0003	3>4
				Wistar	0.0881	0.117	3139	0.75	0.45	1	1	0.85	0.58	
			5. aging	LE	1.50	0.392	3139	3.81	0.0001	*0.022	*0.0028	*0.0018	*0.0003	3>5
		4. adult	5. aging	LE	0.0812	0.252	3139	0.32	0.75	1	1	0.85	0.81	
	M	1. neonat	2. prewean	SD	-0.619	0.217	3139	-2.86	0.0043	0.34	0.060	0.060	*0.0080	2>1
				Wistar	-0.0820	0.352	3139	-0.23	0.82	1	1	0.85	0.85	
			3. prepub	SD	-0.824	1.10	3139	-0.75	0.45	1	1	0.85	0.58	
				Wistar	0.405	0.344	3139	1.18	0.24	1	1	0.85	0.33	
			4. adult	SD	0.723	1.08	3139	0.67	0.50	1	1	0.85	0.58	
				Wistar	1.39	0.407	3139	3.41	0.0007	0.084	*0.011	*0.011	*0.0014	1>4
		2. prewean	3. prepub	SD	-0.205	1.11	3139	-0.18	0.85	1	1	0.85	0.85	
				Wistar	0.487	0.333	3139	1.46	0.14	1	1	0.85	0.24	
			4. adult	SD	1.34	1.10	3139	1.23	0.22	1	1	0.85	0.33	
				Wistar	1.47	0.398	3139	3.69	0.0002	*0.034	*0.0034	*0.0032	*0.0004	2>4
		3. prepub	4. adult	LE	1.95	0.269	3139	7.24	<.0001	*<.0001	*0.0028	*0.0018	*0.0003	3>4
				SD	1.55	0.214	3139	7.24	<.0001	*<.0001	*0.0028	*0.0018	*0.0003	3>4
				Wistar	0.981	0.390	3139	2.51	0.012	0.60	0.16	0.16	*0.021	3>4
P4	F	1. neonat	2. prewean	SD	-0.371	0.131	3326	-2.83	0.0047	0.39	0.099	0.099	*0.011	2>1
				Wistar	-0.371	0.263	3326	-1.41	0.16	1	1	0.81	0.24	
			3. prepub	SD	-0.999	0.141	3326	-7.09	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	3>1
				Wistar	-0.627	0.296	3326	-2.12	0.034	0.90	0.62	0.5600	0.060	
			4. adult	SD	-1.66	0.125	3326	-13.34	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>1
				Wistar	-1.80	0.265	3326	-6.81	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>1
			5. aging	SD	-1.53	0.254	3326	-6.04	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	5>1
				Wistar	-1.89	0.349	3326	-5.41	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	5>1
		2. prewean	3. prepub	SD	-0.628	0.137	3326	-4.59	<.0001	*0.0011	*0.0036	*0.0023	*0.0003	3>2
				Wistar	-0.257	0.214	3326	-1.2	0.23	1	1	0.81	0.30	
			4. adult	SD	-1.29	0.123	3326	-10.49	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>2
				Wistar	-1.43	0.175	3326	-8.18	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>2
			5. aging	SD	-1.16	0.253	3326	-4.59	<.0001	*0.001	*0.0036	*0.0023	*0.0003	5>2
				Wistar	-1.52	0.286	3326	-5.3	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	5>2
		3. prepub	4. adult	LE	-0.432	1.16	3326	-0.37	0.71	1	1	0.81	0.76	
				SD	-0.664	0.124	3326	-5.36	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>3
				Wistar	-1.18	0.195	3326	-6.04	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>3
			5. aging	LE	0.279	1.16	3326	0.24	0.81	1	1	0.81	0.81	
				SD	-0.534	0.253	3326	-2.11	0.035	0.8988	0.62	0.56	0.060	
				Wistar	-1.26	0.299	3326	-4.22	<.0001	*0.0053	*0.0036	*0.0023	*0.0003	5>3
		4. adult	5. aging	LE	0.710	0.0579	3326	12.26	<.0001	*<.0001	*0.0036	*0.0023	*0.0003	4>5
				SD	0.13	0.222	3326	0.59	0.56	1	1	0.81	0.65	
				Wistar	-0.0824	0.227	3326	-0.36	0.72	1	1	0.81	0.76	
	M	1. neonat	2. prewean	SD	-0.734	0.286	3326	-2.56	0.010	0.61	0.21	0.21	*0.022	2>1
				Wistar	-0.455	0.602	3326	-0.75	0.45	1	1	0.81	0.54	
			3. prepub	SD	-1.54	0.539	3326	-2.85	0.0044	0.38	0.097	0.097	*0.011	3>1
				Wistar	-0.635	0.533	3326	-1.19	0.23	1	1	0.81	0.30	
			4. adult	SD	-1.01	0.416	3326	-2.42	0.015	0.71	0.29	0.29	*0.031	4>1
				Wistar	-1.06	0.503	3326	-2.11	0.035	0.90	0.62	0.56	0.060	
		2. prewean	3. prepub	SD	-0.803	0.484	3326	-1.66	0.097	0.99	1	0.81	0.15	
				Wistar	-0.181	0.436	3326	-0.41	0.68	1	1	0.81	0.76	
			4. adult	SD	-0.274	0.346	3326	-0.79	0.43	1	1	0.81	0.53	
				Wistar	-0.607	0.351	3326	-1.73	0.084	0.99	1	0.81	0.14	
		3. prepub	4. adult	LE	-0.297	1.18	3326	-0.25	0.80	1	1	0.81	0.81	
				SD	0.529	0.383	3326	1.38	0.17	1	1	0.81	0.24	
				Wistar	-0.426	0.320	3326	-1.33	0.18	1	1	0.81	0.25	

Separate analyses run by hormone.

*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 10 (continued). Age Effect: Differences of AGE_CAT(STRAIN*SEX) LSMs

		1. neonat	2. prewean	SD	-1.18	0.0861	2982	-13.65	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	2>1
			3. prepub	SD	-0.391	0.0955	2982	-4.1	<.0001	*0.0056	*0.0056	*0.0021	*0.0009	3>1
			4. adult	SD	-0.293	0.114	2982	-2.58	0.01	0.47	0.47	0.070	0.070	
		2. prewean	3. prepub	SD	0.785	0.101	2982	7.74	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	2>3
				Wistar	1.82	0.199	2982	9.15	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	2>3
	F		4. adult	SD	0.883	0.12	2982	7.36	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	2>4
				Wistar	-0.141	0.172	2982	-0.82	0.41	1	1	1	0.45	
		3. prepub	4. adult	LE	-2.05	0.373	2982	-5.51	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>3
				SD	0.0985	0.13	2982	0.76	0.45	1	1	1	0.45	
				Wistar	-1.96	0.172	2982	-11.4	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>3
T			5. aging	LE	-1.89	0.431	2982	-4.38	<.0001	*0.0016	*0.0016	*0.0021	*0.0009	5>3
		4. adult	5. aging	LE	0.165	0.216	2982	0.76	0.45	1	1	1	0.45	
		1. neonat	2. prewean	SD	-0.547	0.200	2982	-2.73	0.0064	0.36	0.36	0.051	0.051	
			3. prepub	LE	-1.69	0.358	2982	-4.74	<.0001	*0.0003	*0.0003	*0.0021	*0.0009	3>1
				SD	0.237	0.263	2982	0.9	0.37	1	1	1	0.45	
			4. adult	LE	-2.28	0.342	2982	-6.65	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>1
	M			SD	-0.935	0.157	2982	-5.95	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>1
		2. prewean	3. prepub	SD	0.784	0.314	2982	2.49	0.013	0.54	0.54	0.076	0.076	
			4. adult	SD	-0.389	0.237	2982	-1.64	0.10	0.98	0.98	0.51	0.45	
		3. prepub	4. adult	LE	-0.580	0.104	2982	-5.58	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>3
				SD	-1.17	0.214	2982	-5.47	<.0001	*<.0001	*<.0001	*0.0021	*0.0009	4>3

Separate analyses run by hormone.
*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 11. Strain Effect: Differences of STRAIN LSMs

	STRAIN	STRAIN	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P
	SD	Wistar	0.177	0.380	3139	0.47	0.64	0.64
E2	LE	SD	Non-est	.	.	.	.	.
	LE	Wistar	Non-est	.	.	.	.	.
	SD	Wistar	Non-est	.	.	.	.	.
P4	LE	SD	Non-est	.	.	.	.	.
	LE	Wistar	Non-est	.	.	.	.	.
	SD	Wistar	Non-est	.	.	.	.	.
T	LE	SD	Non-est	.	.	.	.	.
	LE	Wistar	Non-est	.	.	.	.	.

Separate analyses run by hormone.
*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 12. Strain Effect: Differences of STRAIN*SEX LSMs

	SEX	STRAIN	STRAIN	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
		SD	Wistar	0.306	0.131	3139	2.34	0.019	0.088	*0.038	*0.038	*0.038	SD>W
	F	LE	SD	Non-est	.	.	.	.	.				
		LE	Wistar	Non-est	.	.	.	.	.				
E2		SD	Wistar	0.0484	0.748	3139	0.06	0.95	1	0.95	0.95	0.95	
	M	LE	SD	Non-est	.	.	.	.	.				
		LE	Wistar	Non-est	.	.	.	.	.				
	F	SD	Wistar	Non-est	.	.	.	.	.				
		LE	SD	Non-est	.	.	.	.	.				
P4		LE	Wistar	Non-est	.	.	.	.	.				
	M	SD	Wistar	Non-est	.	.	.	.	.				
		LE	SD	Non-est	.	.	.	.	.				
		SD	Wistar	Non-est	.	.	.	.	.				
	F	LE	SD	Non-est	.	.	.	.	.				
T		LE	Wistar	Non-est	.	.	.	.	.				
		SD	Wistar	Non-est	.	.	.	.	.				
	M	LE	SD	Non-est	.	.	.	.	.				
		LE	Wistar	Non-est	.	.	.	.	.				

Separate analyses run by hormone.
*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

Table 13. Strain Effect: Differences of AGE_CAT(STRAIN) LSMs

	STRAIN	STRAIN	AGE_CAT	Estimate	Standard Error	DF	t Value	Pr > |t|	Adj P	
E2	SD	Wistar	1. neonatal	0.107	0.578	3139	0.18	0.85	1	
			2. preweaning	0.317	0.585	3139	0.54	0.59	1	
			3. prepubertal	0.393	0.396	3139	0.99	0.32	0.99	
			4. adult	-0.107	0.334	3139	-0.32	0.75	1	
	LE	SD	3. prepubertal	-0.342	0.407	3139	-0.84	0.40	1	
			4. adult	-0.990	0.311	3139	-3.18	0.0015	0.048	SD>LE
	LE	Wistar	3. prepubertal	0.0513	0.442	3139	0.12	0.91	1	
			4. adult	-1.10	0.323	3139	-3.40	0.0007	0.024	
P4	SD	Wistar	1. neonatal	-0.147	0.359	3326	-0.41	0.68	1	
			2. preweaning	-0.00721	0.270	3326	-0.03	0.98	1	
			3. prepubertal	0.490	0.355	3326	1.38	0.17	0.93	
			4. adult	-0.245	0.261	3326	-0.94	0.35	1	
			5. aging	Non-est	.	.	.	.	.	
	LE	SD	3. prepubertal	0.0690	1.15	3326	0.06	0.95	1	
			4. adult	0.366	0.415	3326	0.88	0.38	1	
			5. aging	Non-est	.	.	.	.	.	
	LE	Wistar	3. prepubertal	0.559	1.14	3326	0.49	0.62	1	
			4. adult	0.121	0.399	3326	0.30	0.76	1	
			5. aging	Non-est	.	.	.	.	.	
T	SD	Wistar	2. preweaning	Non-est	.	.	.	.	.	
			3. prepubertal	Non-est	.	.	.	.	.	
			4. adult	-0.715	0.306	2982	-2.33	0.020	0.23	
	LE	SD	1. neonatal	Non-est	.	.	.	.	.	
			3. prepubertal	-0.161	0.316	2982	-0.51	0.61	1	
			4. adult	0.619	0.247	2982	2.50	0.012	0.16	
	LE	Wistar	3. prepubertal	Non-est	.	.	.	.	.	
			4. adult	-0.0959	0.308	2982	-0.31	0.76	1	

Separate analyses run by hormone.

*statistically significant according to Tukey-adjusted p-value, "Adj P," alpha = 0.05.

Table 14. Strain Effect: Differences of AGE_CAT(STRAIN*SEX) LSMs

	SEX	STRAIN	STRAIN	AGE_CAT	Estimate	SE	DF	t Value	Pr > |t|	Adj P (Tukey)	Adj P (Holm)	Adj P (Hoch)	Adj P (FDR)	
E2	F	SD	Wistar	1. neonatal	0.772	0.155	3139	4.98	<.0001	*<.0001	*0.0016	*0.0016	*0.0016	SD>W
				2. preweaning	0.656	0.180	3139	3.64	0.0003	*0.040	*0.0042	*0.0042	*0.0016	SD>W
				3. prepubertal	0.116	0.177	3139	0.65	0.51	1	1	0.98	0.68	
				4. adult	-0.318	0.140	3139	-2.27	0.023	0.78	0.23	0.23	0.053	
		LE	SD	3. prepubertal	0.321	0.356	3139	0.9	0.37	1	1	0.98	0.56	
				4. adult	-0.571	0.209	3139	-2.73	0.0063	0.43	0.082	0.082	*0.025	SD>LE
		LE	Wistar	3. prepubertal	0.437	0.379	3139	1.15	0.25	1	1	0.98	0.44	
				4. adult	-0.889	0.241	3139	-3.69	0.0002	*0.034	*0.0030	*0.0030	*0.0016	W>LE
	M	SD	Wistar	1. neonatal	-0.559	1.14	3139	-0.49	0.63	1	1	0.98	0.71	
				2. preweaning	-0.0221	1.16	3139	-0.02	0.98	1	1	0.98	0.98	
				3. prepubertal	0.670	0.772	3139	0.87	0.39	1	1	0.98	0.56	
				4. adult	0.104	0.653	3139	0.16	0.87	1	1	0.98	0.93	
		LE	SD	3. prepubertal	-1.00	0.612	3139	-1.64	0.10	0.99	0.90	0.90	0.20	
				4. adult	-1.41	0.530	3139	-2.66	0.0079	0.48	0.095	0.09	*0.025	SD>LE
		LE	Wistar	3. prepubertal	-0.334	0.685	3139	-0.49	0.63	1	1	0.98	0.71	
				4. adult	-1.30	0.535	3139	-2.44	0.015	0.66	0.16	0.16	*0.040	W>LE
P4	F	SD	Wistar	1. neonatal	0.413	0.441	3326	0.94	0.35	1	1	0.99	0.74	
				2. preweaning	0.413	0.394	3326	1.05	0.29	1	1	0.99	0.70	
				3. prepubertal	0.785	0.401	3326	1.96	0.051	0.95	0.96	0.96	0.34	
				4. adult	0.271	0.338	3326	0.8	0.42	1	1	0.99	0.80	
				5. aging	0.0586	0.443	3326	0.13	0.89	1	1	0.99	0.99	
		LE	SD	3. prepubertal	-0.0318	1.14	3326	-0.03	0.98	1	1	0.99	0.99	
				4. adult	-0.264	0.405	3326	-0.65	0.51	1	1	0.99	0.82	
				5. aging	-0.845	0.455	3326	-1.86	0.064	0.97	1	0.99	0.34	
		LE	Wistar	3. prepubertal	0.753	1.16	3326	0.65	0.51	1	1	0.99	0.82	
				4. adult	0.00687	0.413	3326	0.02	0.99	1	1	0.99	0.99	
				5. aging	-0.786	0.465	3326	-1.69	0.091	1	1	0.99	0.34	
	M	SD	Wistar	1. neonatal	-0.707	0.566	3326	-1.25	0.21	1	1	0.99	0.67	
				2. preweaning	-0.428	0.371	3326	-1.15	0.25	1	1	0.99	0.67	
				3. prepubertal	0.195	0.588	3326	0.33	0.74	1	1	0.99	0.97	
				4. adult	-0.761	0.399	3326	-1.91	0.057	0.96	1	0.99	0.34	
		LE	SD	3. prepubertal	0.170	1.25	3326	0.14	0.89	1	1	0.99	0.99	
				4. adult	0.996	0.561	3326	1.77	0.076	0.98	1	0.99	0.34	
		LE	Wistar	3. prepubertal	0.364	1.21	3326	0.3	0.76	1	1	0.99	0.97	
				4. adult	0.235	0.509	3326	0.46	0.64	1	1	0.99	0.94	
T	F	SD	Wistar	2. preweaning	0.390	0.588	2982	0.66	0.51	1	1	0.62	0.55	
				3. prepubertal	1.42	0.589	2982	2.41	0.016	0.60	0.13	0.13	*0.038	SD>W
				4. adult	-0.634	0.561	2982	-1.13	0.26	1	1	0.62	0.39	
		LE	SD	3. prepubertal	-1.12	0.501	2982	-2.23	0.026	0.73	0.15	0.15	*0.044	SD>LE
				4. adult	1.034	0.382	2982	2.71	0.0068	0.38	0.075	0.075	*0.032	LE>SD
		LE	Wistar	3. prepubertal	0.303	0.618	2982	0.49	0.62	1	1	0.62	0.62	
				4. adult	0.400	0.508	2982	0.79	0.43	1	1	0.62	0.52	
		SD	Wistar	4. adult	-0.796	0.242	2982	-3.29	0.001	0.092	*0.012	*0.012	*0.012	W>SD
	M	LE	SD	1. neonatal	-1.14	0.428	2982	-2.65	0.008	0.41	0.080	0.080	*0.032	SD>LE
				3. prepubertal	0.796	0.320	2982	2.49	0.013	0.54	0.12	0.12	*0.038	LE>SD
				4. adult	0.204	0.221	2982	0.92	0.36	1	1	0.62	0.48	
		LE	Wistar	4. adult	-0.592	0.257	2982	-2.31	0.021	0.68	0.15	0.15	*0.042	W>LE

Separate analyses run by hormone.

*statistically significant according to p-value adjusted by method in parentheses ("Tukey" = Tukey HSD, "Holm" = step-down Holm, "Hoch" = step-up Hochberg, "FDR" = False discovery rate), alpha = 0.05.

References

Bailey, J. A., Nephew, K. P. (2002). Strain differences in tamoxifen sensitivity of Sprague-Dawley and Fischer 344 rats. *Anti-Cancer Drugs*, *13*(9), 939-947.

Dimauro C, Bonelli P, Nicolussi P, et al., (2008). Estimating clinical chemistry reference values based on an existing data set of unseslected animals. *The Veterinary Journal* 178:278-281.

Diel, P., Laudenbach, U., Smolnikar, et al., (2001a). Bisphenol A: Morphological and molecular uterine and mammary gland reactions in different strains of the rat (Wistar, Sprague-Dawley, Da/Han). *Toxicologist* **60**(1), 296 (Abstract).

Festing, M. F. (2010). Inbred strains should replace outbred stocks in toxicology, safety testing, and drug development. *Toxicologic Pathology*,*38*(5), 681-690.

Hedrich, H. J. (2000). History, strains and models. *The Laboratory Rat. Academic Press, London*, 3-8.

Howdeshell, K. L., Furr, J., Lambright, C. R., et al., (2008). Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male long evans hooded rat. *Toxicological Sciences*, *102*(2), 371-382.

Kacew, S., Festing, M. F. W. (1999). Role of Rat Strain in the Differential Sensitivity to Pharmaceutical Agents and Naturally Occurrin Substances. *Central European Journal of Occupational and Environmental Medicine*,*5*(3/4), 201-231.

Kwon, S., Stedman, D. B., Elswick, B. A., et al., (2000). Pubertal development and reproductive functions of Crl: CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. *Toxicological Sciences*, *55*(2), 399-406.

Long, X., Steinmetz, R., Ben-Jonathan, N., et al., (2000). Strain differences in vaginal responses to the xenoestrogen bisphenol A. *Environmental Health Perspectives*, *108*(3), 243.

O'Connor, J. C., Frame, S. R., Davis, L. G., et al., (1999). Detection of the environmental antiandrogen p, p-DDE in CD and long-evans rats using a tier I screening battery and a Hershberger assay. *Toxicological Sciences*,*51*(1), 44-53.

Pandey, J., Gould, K. A., McComb, R. D., et al., (2005). Localization of Eutr2, a locus controlling susceptibility to DES-induced uterine inflammation and pyometritis, to RNO5 using a congenic rat strain. *Mammalian Genome*, *16*(11), 865-872.

Rachdaoui, N., Sarkar, D. K. (2013). Effects of alcohol on the endocrine system. Endocrinology and metabolism clinics of North America, 42(3), 593-615.

Spearow, J. L., Doemeny, P., Sera, R., et al., (1999). Genetic variation in susceptibility to endocrine disruption by estrogen in mice. *Science*, *285*(5431), 1259-1261.

Steinmetz, R., Brown, N. G., Allen, D. L., et al., (1997). The Environmental Estrogen Bisphenol A Stimulates Prolactin Release in Vitro and in Vivo 1. *Endocrinology*, *138*(5), 1780-1786.

Strom, D. J., Stansbury, P. S. (2000). Determining parameters of lognormal distributions from minimal information. AIHAJ-American Industrial Hygiene Association, 61(6), 877-880.

Turner P.V., Brabb T, Pekow C, et al., Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. *Journal of the American Association for Laboratory Animal Science : JAALAS*. 2011;50(5):600-613.

Tyl, R. W., Myers, C. B., Marr, M. C., et al., (2002). Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. *Toxicological Sciences*,*68*(1), 121-146.

Wilkinson, J. M., Halley, S., Towers, P. A. (2000). Comparison of male reproductive parameters in three rat strains: Dark Agouti, Sprague-Dawley and Wistar. *Laboratory Animals*, *34*(1), 70-75.

You, L., Casanova, M., Archibeque-Engle, S., et al., (1998). Impaired male sexual development in perinatal Sprague-Dawley and Long-Evans hooded rats exposed in utero and lactationally to p, p'-DDE. *Toxicological Sciences*, *45*(2), 162-173.

Chapter 2: Effect of Perinatal Exposure to Bisphenol-A on Sex Hormone Levels in Young Rats

Introduction

BPA (bisphenol A) is a chemical compound with known weak estrogenic activity that is commonly used as an additive in polycarbonate plastic products in a wide variety of consumer products. In 2006, an expert panel met in Chapel Hill, NC, USA, to discuss the current consensus and gaps in knowledge surrounding the effects of BPA (vom Saal, et al., 2007). They reaffirmed that both the pharmacokinetics of BPA and the sensitivity of the body to endocrine disruptors vary with life stage, with some time-points comprising important developmental windows. Due to life stage discrepancies among studies, they concluded, that further longitudinal studies are needed to properly evaluate the comprehensive effects of BPA exposure during development on the reproductive and other systems throughout the organism's lifespan. Their conclusions were updated by Vandenberg, et al., (2013), who concluded that reproducible effects in animals are produced at doses 0.1 to 0.0001 lower than the current LOAEL of 50 mg/kg/day. Heindel (2005) pointed out that any particular toxicant, depending on the time or duration of exposure, has the potential to exhibit a period of latency for any amount of time before effects are able to be observed, with the onset of these effects potentially occurring as early as the neonatal period or as late as adulthood.

Numerous studies have demonstrated unfavorable effects of developmental BPA exposure at environmentally relevant dosages ("relevant-dose") on adult reproductive parameters. Contrastingly, few studies have evaluated effects at other time points, including infantile development, which spans from parturition to PND 10 in the rat. The Developmental Origin of Health and Disease (DOHaD) theory is that exposure to environmental challenges

during early periods of development, during which tissue usually possess a higher degree of plasticity, can influence the risk of diseases later in life (Wadhwa, et al., 2009). Exposure to endocrine disruptors during childhood is of special concern because of the effects that hormonally-active substances can have on neurologic, endocrine, and reproductive development (Diamanti-Kandarakis, et al., 2009).

Newborn rats are less developmentally mature than humans. Thus, post-natal day (PND) 10 rats are used for comparison to human infants at birth (Clancy 2007, Dobbing and Sands 1979, Andrews and Fitzgerald 1997). PND 10 is also a critical time for neuroendocrine and reproductive development in rats, as the hypothalamus and pituitary are comparatively much more sensitive to sex hormones at this age (Andrews, et al., 1981). Thus, potentially significant pathological effects could result from even small changes during that period. Here, we investigated the effect of orally administered BPA during the perinatal period on sex hormones in both male and female infantile (PND 10) rats.

Methods

Housing and Diet

Adult Long-Evans rats (PND 80 – 100) were obtained from Harlan (Indianapolis, IN). Care was taken to reduce BPA exposure in the housing environment by providing reverse osmosis water in glass or polysulfone drinking bottles and using polysulfone cages and beta-chip bedding. The Harlan 2020X diet was chosen because it contains minimal amounts of phytoestrogens, which are well-known endocrine disruptors. During the 2 weeks prior to breeding, 2 to 3 females were housed per cage, and males were housed singly to prevent aggressive behavior. Handling of rats was performed a few times prior to the start of the study to acclimate rats to the procedure, and female breeders were trained to receive half a cookie (Newman's Own Arrowroot flavor, Westport, CT) covered with tocopherol-stripped corn oil (100 µL) once returned to their cage. This particular cookie brand was chosen because they have been shown to contain no measurable levels of BPA. Breeding was initiated by cohabitating one male and one female in a metal cage with daily monitoring. Females were considered pregnant upon the presence of a sperm plug. Dams were then housed individually for the gestation and lactation periods.

BPA exposure

Our study involved exposure to BPA perinatally with both a prenatal and a postnatal exposure. Indeed, our original study was tailored towards BPA developmental neurotoxicity. Brain development in the rat during PND 0-9 mimics development during the third trimester of pregnancy in humans (Clancy, et al., 2007, Dobbing and Sands 1979). The postnatal dosing

period was based on recent evidence that lactation results in very low BPA exposure in rat pups when dams are dosed orally (Doerge, et al., 2010).

For the prenatal period, dosing of the dams started on gestational day (GD) 3 and stopped upon parturition (GD 21 or PND 0). Treatment groups were evenly distributed with rats so that there were no differences in overall average body weight. BPA powder was suspended in tocopherol-stripped corn oil vehicle to make 0, 0.1, and 1 mg BPA/mL oil suspensions that were re-mixed daily with a stir bar to ensure homogeneity. Vehicle or BPA solution (0.4 µL/g of body weight) was pipetted onto half a cookie to reach the following doses: 0, 40, and 400 ug BPA per kg of body weight. Cookies were allowed to dry before being given to the dams. All rats consumed the entire half cookie within 5 minutes.

To start the postnatal period, litter sizes and sex ratios were recorded on PND 1. On PND 2, litters were culled to 7-10 pups per dam with as equal a sex ratio as possible between litters. Stocks of 0, 0.02, and 0.2 mg BPA/mL oil were prepared prior to each cohort and re-mixed daily. Dosing of the pups started on PND 2 and continued to PND 10, at which point dosing was stopped one hour prior to sacrifice. Each pup was weighed daily and two separate oral doses, one right after the other, for a total of 2 µL/g of body weight, were pipetted into their mouth to reach the same dose received by the dams. Both oral and non-oral dosing of neonatal-infantile pups have been demonstrated to not differ in active levels of BPA due to the low activity of liver enzymes (Taylor, et al., 2008). During dosing, pups were separated from the dam for 10-15 minutes.

Sample collection and processing

At PND 10, one hour after BPA administration, pups were euthanized and trunk blood was collected from one female and one male per litter in glass tubes and immediately placed in a refrigerator at 4°C. Within 1 hour of collection, blood samples were spun at 3500 rpm for 10 minutes and the plasma fraction was aliquoted and stored at -80°C until further processing. Samples from 6 males and 8 females were collected for the vehicle control group (BPA 0), 9 males and 9 females for the 40 μg/kg BPA group (BPA 40), and 10 males and 9 females for the 400 μg/kg BPA group (BPA 400).

FSH/LH measurement

FSH and LH were measured using a multi-analyte Luminex kit (Millipore, *St. Charles, MO,* USA) according to the manufacturer's instructions. Briefly, plasma samples were diluted with serum matrix in a 1:2 dilution. Standards, quality controls, and samples were incubated overnight, after which antibodies and Streptavidin-Phycoerythrin were added. The plate was read within 20 minutes on a Luminex plate reader (BioPlex 200 with Bio-Plex Manager Software 4.0; BioRad, *Hercules, CA,* USA).

Steroid extraction

Steroid extraction was carried out to reduce background interference in the ELISA kits (Tate and Ward 2004). Extraction was performed by adding ethyl acetate to plasma in glass culture vials (5:1 ratio) and thoroughly mixing by vortexing. Solutions were then centrifuged at 3000 rpm for 10 minutes at room temperature and frozen at -20°C overnight. The upper liquid

organic phases were then transferred into new glass vials for evaporation using a rotary evaporator at 40°C for 3 minutes. Dried samples were resuspended in steroid-free serum (IBL America, Minneapolis, MN, USA), vortexed thoroughly, and stored at -20°C until ELISA testing.

Steroid sex hormone ELISA

Testosterone, progesterone, and estradiol were measured in extracted samples using commercial ELISA kits (IBL America, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples were undiluted with the exception of twelve that were diluted in steroid-free serum after giving saturated signals during the original estradiol analysis.

Statistical analysis

Statistical analyses were carried out using SAS 9.3 (SAS Institute Inc., Cary, NC). Preliminary analyses for each hormone included summary statistics, probability plots, and the Shapiro-Wilk test and Anderson-Darling test to determine if the distribution of the data for the hormone was Normal (Gaussian). Skewness and kurtosis (Mardia 1970), and a test of multinormality (Henze and Zirkler 1990), were used to determine multivariate normality of the five hormones. The M test (Box 1949) was used to determine homogeneity of the covariance matrices for males versus females. Spearman rank correlations between all hormones were determined for female pups, male pups, and all pups. Hormone concentrations of each variable, LH, FSH, testosterone, progesterone, and estradiol, were standardized to a mean of zero and standard deviation of 1 so that all categories could be compared on the same unit-less scale.

The same sample from each pup (one of each sex from each dam) was analyzed for all hormones (LH, FSH, T, P4, E2) on the pup (within-subject design). Hormone concentrations were normalized using the log modulus transformation (John and Draper 1980). The log modulus transformation of a value, y, is: $\text{sign}(y) \times \log_{10}(y + 1)$. Transformed values were analyzed as mixed-effects ANOVA (Proc GLIMMIX), where dam was a random factor and hormone, treatment, and sex were fixed factors. All statistical values are reported and discussed in terms of the transformed values and adjusted P-values unless specifically stated otherwise.

The male:female ratio is reported in arithmetic units, and was estimated using an online calculator from GraphPad (2016) from the reported mean and standard error of studies that measured sex hormones in PND 9-11 rats. Ratios were evaluated rather than point hormone values due to large differences in the scales of the mean concentrations between studies, most likely due to differences in the cross-reactivity and sensitivity of the specific antibodies, and other differences in detection methods.

P-values for selected pairwise comparisons “hormone x sex” and “hormone x treatment x sex” were Bonferroni-adjusted to maintain a family-wise Type I error rate of $\alpha = 0.05$. The selected comparisons were between-treatments within hormone and sex, or between sexes within hormone and treatment. Other pairwise comparisons were not selected, e.g., between hormones and concurrently between sexes and treatments.

Results

Summary statistics for all hormones are in Table 15.

Sex differences

Hormone, sex, and their interaction (hormone × sex), were statistically significant ($p < 0.05$) (Table 16). The treatment x sex and hormone x treatment x sex interactions were not statistically significant. These interactions are averaged over all hormones, which does not preclude statistical significance for some combinations discussed below.

Mean hormone concentrations averaged across treatments for each sex were statistically significant for the gonadotropins, FSH and LH ($p < 0.01$), but not for the steroid hormones, estradiol (E2), progesterone (P4), or testosterone (T) ($p > 0.5$) (Table 17, Figure 1, 2). Within LH, all treatment groups showed statistically significant differences between males and females ($p < 0.01$) (Table 18, Figure 1). For FSH, the BPA 40 and BPA 400 groups showed statistically significant differences between males and females ($p < 0.01$) (Table 18, Figure 1), but the control group (BPA 0) was not significant ($p = 0.017$, $\alpha = 0.01$).

Treatment effect

Treatment, and the treatment x sex x hormone interaction, and hormone x sex interactions, were not statistically significant (all $p > 0.3$) (Table 16). Because of this, treatment was assumed to have no statistically significant influence on hormone concentrations and, thus, further analyses of pairwise comparisons for treatment (in Appendices 3-5) were not of interest. For hormone x treatment x sex comparisons, all $p > 0.4$ (Table 24).

Discussion

Sex differences

Females showed higher baseline (untreated) FSH and LH concentrations than males (Table 17, 4, Figure 1). This was not the case for estradiol, testosterone, or progesterone (Table 17, Figure 2). Sex differences for FSH and LH are consistent with previous rat studies that reported hormone levels in both sexes. This study, as in previous studies, found that levels of FSH in males were lower than females (males were 39% of females, compared to the 28% which we found) (Table 18, 5). The sex ratio for FSH that we found was consistent with the literature, with the previous studies' combined confidence interval overlapping our confidence interval by 100%. Male LH levels measured in previous studies were 51% of female values, compared to the 53% that we found (Table 19). Thus, the particular M:F ratio computed for FSH and LH of this study are consistent with the M:F ratios found by previous studies.

In contrast to the gonadotropins, steroid sex hormones were not different between males and females for estradiol, progesterone, or testosterone (Table 17). The sex ratios that we found for the three hormones were consistent with the previous literature, with our estradiol results overlapped by 63% of the previous studies' combined confidence interval, progesterone overlapped by 100%, and testosterone overlapped by 66% (Table 19). The lack of a sex difference in steroidal sex hormones for infantile/preweaning rats is also consistent with the findings of the first chapter of this thesis (see discussion of sex differences in Chapter 1).

Treatment effect

Other than two studies of note, Kato, et al., 2006 and Ramos, et al., 2003, there are limited published data on the effects of "relevant-dose," perinatal BPA exposure on the sex hormone profiles of infantile rats. Here, we define relevant-dose exposure as below 500 μg/kg body weight oral BPA, based on studies in mice and rhesus monkeys that showed that oral exposure to 400 μg/kg body weight BPA leads to systemic blood levels comparable to epidemiologic studies that measured human blood and urine levels of BPA (Taylor, et al., 2011). In the Kato study, male rats were postnatally exposed to relevant-dose BPA and hormone levels were determined at PND 10. No statistical difference was found on body weight, mRNA levels of steroidogenic enzymes, mRNA levels of steroid hormone receptors in the testes, or peripheral levels of testosterone. This is in contrast to the Ramos study, where relevant-dose BPA was limited to gestational exposure. Peripheral testosterone was found to be statistically significantly increased in PND 15 male rats. The conflicting outcomes of the two studies could have resulted from a number of reasons related to different laboratory practices, exposure timing, rat strain, assay methodology, and the 5-day age difference.

Relevant-dose, perinatally administered BPA alters gonadotropin levels in weaned and adult males (Bai, et al., 2011, Gamez, et al., 2014, Sadowski, et al., 2014), as well as steroid sex hormones (Chen, et al., 2014, Bai, et al., 2011, Aloisi, et al., 2002). Gonadotropins in female weanling or adult rats were also affected by relevant-dose, perinatal BPA (Gamez, et al., 2015, Sadowski, et al., 2014), as were the steroid sex hormones (Gamez, et al., 2015, Aloisi, et al., 2002, Durando, et al., 2011). However, studies have also reported no effects in both genders of rats of post-juvenile age that had been exposed perinatally exposed to relevant-dose BPA

(Akingbemi, et al., 2004, Howdeshell, et al., 2007, Ramos, et al., 2003, Ferguson, et al., 2011, 2014, Sadowski, et al., 2014, Gamez, et al., 2014, 2015, Nanjappa, et al., 2012, Kobayashi, et al., 2012, Aloisi, et al., 2002, Chen, et al., 2014, Durando, et al., 2011, Monje, et al., 2007). In summary, there are conflicting reports of the effects of exposure to BPA in the infantile period, as well as in later stages, which makes it difficult to reach a consensus about reproducibility of relevant dose exposure at pre-adult life stages in the rat when the subject has been brought to discussion (Melnick, et al., 2002).

In the present study, perinatal BPA treatment of 40 or 400 μg/kg bw did not significantly affect any of the hormone levels tested in either males or females. These results are consistent with Kato, et al., (2006) and the gonadotropin results of the Ramos, et al., (2003). The absence of an effect on infantile rat hormone concentrations does not predict the absence of observable effects in these animals as the immature hypothalamic-pituitary-gonadal system associated with sexual activity matures. Although the hypothalamic-pituitary unit is substantially developed at PND 10, it is not until the second or third week of life, in the middle-to-late infantile period in the female rat, that the gonadal unit—the gonadotropin-responsive secondary follicles—begin to form and begin ovarian-mediated secretion of estradiol (Prevot 2015). Thus, the absence of significant differences in female steroid hormone levels in fetal-neonatal rats exposed to BPA relative to unexposed pups could be due to the underdevelopment of the ovaries at this exposure period. This hypothetic explanation is further supported by the absence of significant sexually dimorphic sex steroid profiles in the control pups of this study, which would normally emerge in adulthood (see Chapter 1). The absence of effects on male steroid levels can possibly

be attributed possibly to the low, still growing, population of postnatal Leydig cells (Prevot 2015).

Study Limitations

The pulsatile nature of the secretion of hormones could potentially result in a wide variability in hormone levels and affect the statistical power of our study, especially in combination with the low number of animals that were used for each treatment group. In infantile rats, serial blood draws from the same subject are impractical given the volume of sample needed for the assays (for assays in duplicate: 20 µl for combined FSH/LH panel, 50 µl each for T and P4 ELISAs, and 200 µl for E2 ELISA) relative to the size and total blood volume (< 1 ml) of each animal. Compensation by including a number of individual subjects from different dams or the same dam in a pooled sample, (along with appropriate statistical analysis to account for subject), are the common alternatives to serial draws.

Calabrese and Baldwin (2001) stressed the importance of using a wide range of dosages in order to detect non-monotonic dose-response curves. Although our study covered a wide range within the scope of dosages relevant to average human exposure, additional (more closely-spaced) doses within that interval would facilitate defining the shape of the dose-response curve.

Conclusions

Based on a review of the very few relevant BPA studies, our results are consistent with some of what has been reported after relevant-dose BPA exposure in the infantile, juvenile,

peripubertal, and adult periods in rats. However, the literature is limited and sometimes contradictory for relevant-dose perinatal exposure in rats. Although relevant-dose perinatal BPA exposure in rats may not affect sex hormone levels in infantile rats (from what we observed here), we cannot say that BPA does not affect infantile physiology in other ways, possibly through epigenetic mechanisms, that lead to immediate or delayed effects on other reproductive (and non-reproductive) parameters as the rats mature. Further exploration of the effects of both relevant and higher dosages of BPA on a wider range of reproductive parameters within this critical developmental time in the infantile period of the rat, combined with similar observations at the later stages of development, would provide vital insight.

Tables and Figures

Table 15. Summary statistics by hormone and sex

		Male			*Female*		
		BPA 0	**BPA 40**	**BPA 400**	**BPA 0**	**BPA 40**	**BPA 400**
	Statistic	(n = 6)	(n = 9)	(n = 10)	(n = 8)	(n = 9)	(n = 9)
FSH (pg/ml)	*Mean*	5568.6	6590.5	6684.7	19611.1	23903.4	23958.7
	Median	4269.4	6271.7	6721.2	18401.2	19139.7	21558.5
	Min	2809.2	2214.6	3750.5	14634.1	14785.0	12837.2
	Max	12299.3	13377.3	9789.8	26336.5	49713.6	36748.4
	Std Dev	3518.7	3334.4	1928.4	4449.3	11465.2	9312.6
	Std Error	1436.5	1111.5	609.8	1573.1	3821.7	3104.2
LH (pg/ml)	*Mean*	453.6	406.3	405.9	855.6	1072.6	1039.7
	Median	155.6	97.0	132.1	565.6	618.3	1156.0
	Min	-46.8	-28.6	7.6	310.2	253.6	207.8
	Max	2258.1	1657.9	1378.7	2073.0	2376.4	1981.1
	Std Dev	888.4	576.5	475.2	654.2	814.8	620.5
	Std Error	362.7	192.2	150.3	231.3	271.6	206.8
E2 (pg/ml)	*Mean*	239.5	248.4	300.3	765.7	394.5	445.7
	Median	188.8	208.8	199.5	311.5	228.2	194.6
	Min	48.5	182.4	133.2	52.0	160.0	131.2
	Max	631.6	596.6	606.1	2101.7	1243.5	2347.9
	Std Dev	201.0	131.1	194.9	817.8	364.0	716.2
	Std Error	82.1	43.7	61.6	289.1	121.3	238.7
P4 (ng/ml)	*Mean*	0.32	0.27	0.43	0.36	0.31	0.30
	Median	0.20	0.20	0.30	0.30	0.21	0.27
	Min	0.06	0.04	0.10	0.04	0.02	0.03
	Max	1.08	0.77	1.08	0.88	0.98	0.67
	Std Dev	0.38	0.23	0.33	0.27	0.30	0.20
	Std Error	0.16	0.08	0.10	0.10	0.10	0.07
T (ng/ml)	*Mean*	0.50	0.34	0.52	0.23	0.21	0.21
	Median	0.51	0.27	0.39	0.19	0.17	0.19
	Min	0.06	0.04	0.21	0.04	0.02	0.03
	Max	0.98	0.88	1.08	0.91	0.50	0.51
	Std Dev	0.35	0.25	0.33	0.28	0.16	0.13
	Std Error	0.14	0.08	0.10	0.10	0.05	0.04

Table 16. Main effects and interactions of sex and hormone on mean estimated hormone concentration.

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Hormone	4	100	737	*<0.01
Sex	1	20	27	*<0.01
Hormone*Sex	4	80	15	*<0.01
Treatment	2	25	1	0.35
Treatment*Sex	2	20	0.48	0.63
Hormone*Treatment	8	100	0.44	0.90
Hormone*Treatment*Sex	8	80	0.31	0.96

*statistically significant according to critical value, $\alpha = 0.05$

Table 17. Effects of sex on mean estimated hormone concentration by hormone (FSH, LH, E2, P4, T) averaged over all treatments

Differences of Least Squares Means (Hormone x Sex)

Hormone	Estimate	Standard Error	DF	t Value	Pr > |t|	
FSH	0.36	0.078	80	4.6	*<0.01	F > M
LH	0.62	0.078	80	8.0	*<0.01	F > M
E2	0.017	0.078	80	0.21	0.83	
P4	-0.045	0.078	80	-0.58	0.56	
T	-0.047	0.078	80	-0.61	0.55	

*statistically significant according to Bonferroni-adjusted critical value, $\alpha = 0.05 / 5 = 0.01$

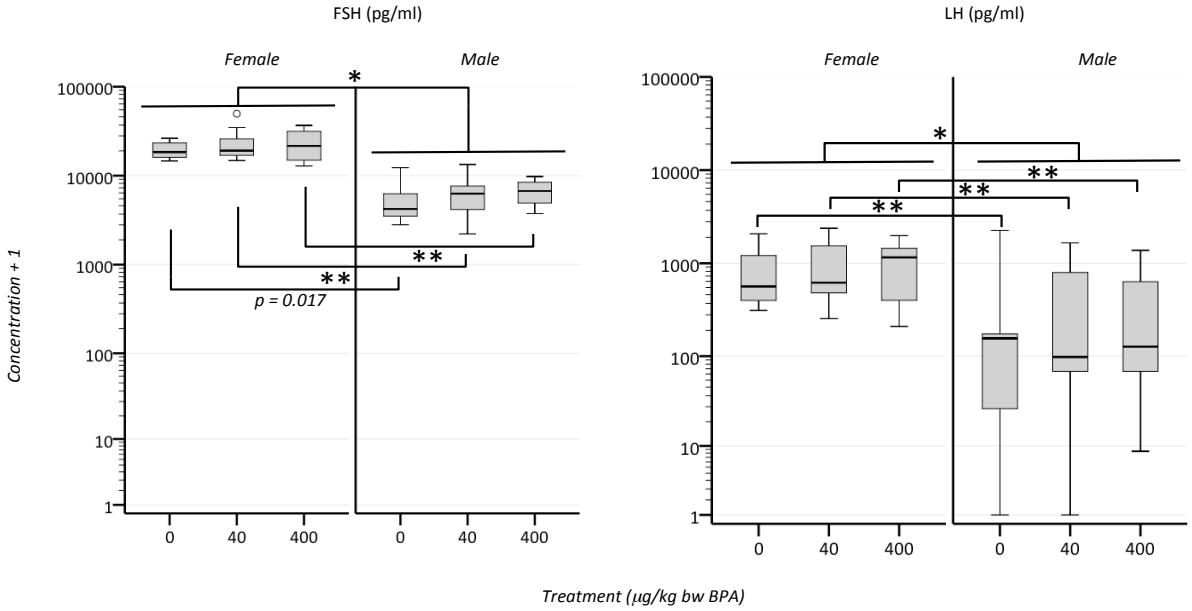
Table 18. Effects of sex on mean estimated hormone concentration by hormone (FSH, LH) and treatment (BPA 0, BPA 40, BPA 400)

Differences of Least Squares Means (Hormone x Treatment x Sex)

Hormone	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|	
FSH	BPA 0	0.38	0.15	80	2.4	0.017	
	BPA 40	0.36	0.13	80	2.9	*<0.01	F > M
	BPA 400	0.34	0.12	80	2.8	*<0.01	F > M
LH	BPA 0	0.77	0.15	80	5.0	*<0.01	F > M
	BPA 40	0.67	0.13	80	5.2	*<0.01	F > M
	BPA 400	0.43	0.12	80	3.6	*<0.01	F > M

*statistically significant according to Bonferroni-adjusted critical value, $\alpha = 0.05 / 6 = 0.008\overline{3} \approx 0.01$

Figure 1. Gonadotropins



*Significant sex effect (sex x hormone interaction); Bonferroni-adjusted critical value, α = 0.05 / 5 = 0.01
**Significant sex effect (sex x hormone x treatment interaction); Bonferroni-adjusted critical value, α = 0.05 / 6 = $0.008\overline{3}$ ≈ 0.01

Figure 2. Steroid hormones

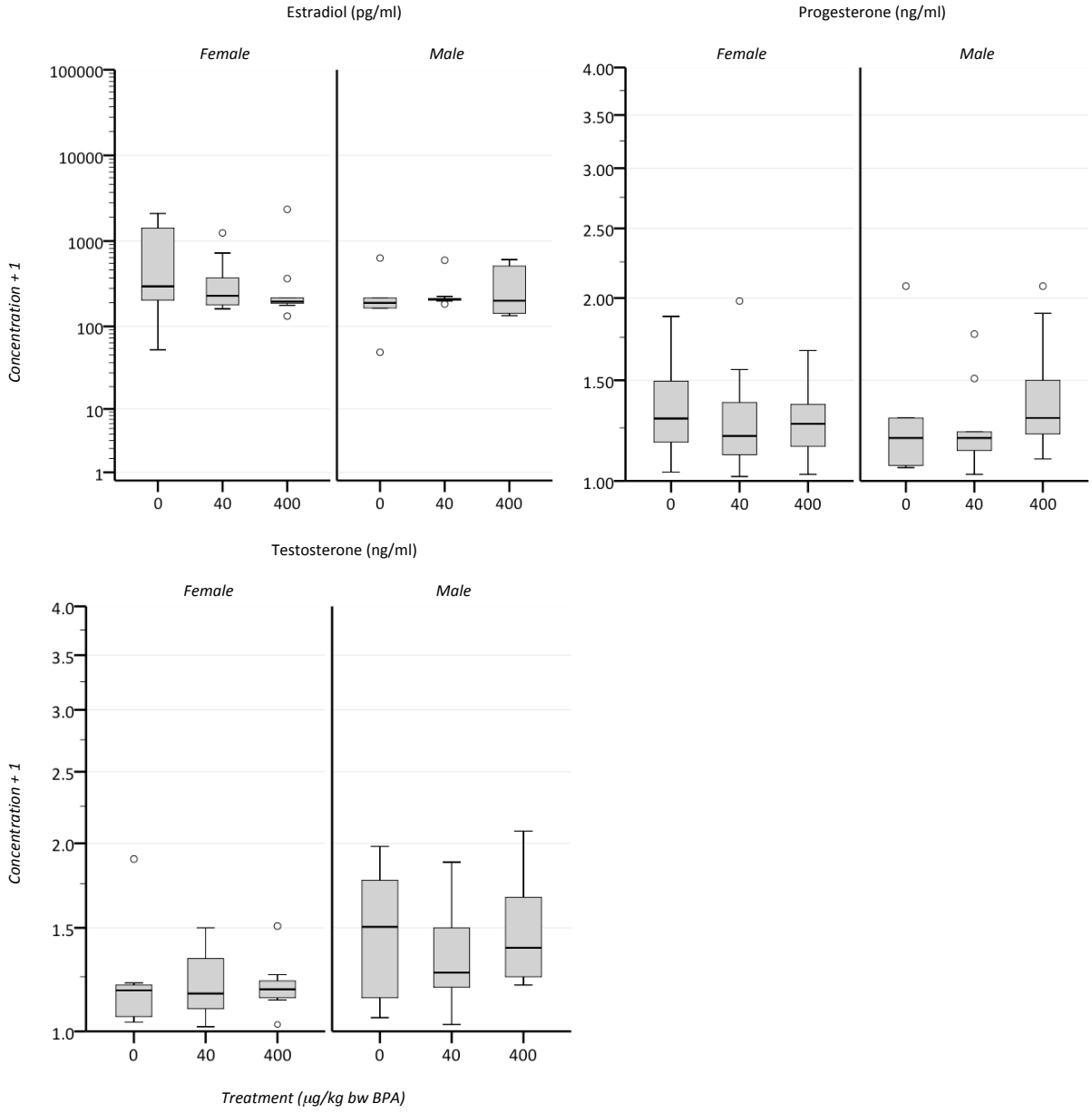


Table 19. Hormones sex ratios around PND 10-11 in the literature

	Source	Kit, antibody	Strain	n	Male	Female	M:F ratio	90% CI	% overlap with previous studies
FSH (ng/ml)	Döhler & Wuttke 1974	RIA, NIAMD	SD	3-8	175 ± 60	892 ± 244	0.2	0.07-0.45	
	Döhler & Wuttke 1975	RIA, NIAMD	SD	8-15	281 ± 13	752 ± 111	0.37	0.29-0.51	
	Dahl, et al., 1988	RIA, NIDDKD	SD	3-4	8.6 ± 2	21 ± 3.9	0.41	0.21-0.73	
	Kamberi, et al., 1980	RIA	W	5	376 ± 27	880 ± 32	0.43	0.37-0.49	
	Ojeda & Ramírez 1972	RIA, NIAMD	W	6-8	454 ± 106	860 ± 118	0.53	0.30-0.83	
						Avg:	0.39	0.07-0.83	
	Current study	EIA	LE	6-8	5.57 ± 1.44	19.61 ± 1.57	0.28	0.15-0.43	100%
LH (ng/ml)	Döhler & Wuttke 1974	RIA, NIAMD	SD	3-8	30 ± 7	550 ± 155	0.05	0.03-0.12	
	Döhler & Wuttke 1975	RIA, NIAMD	SD	8-15	34 ± 6	89 ± 39	0.38	0.20-1.57	
	Kamberi, et al., 1980	RIA	W	5	110 ± 8	265 ± 80	0.42	0.26-0.95	
	Ojeda & Ramírez 1972	RIA, NIAMD	W	6-8	417 ± 43	343 ± 88	1.22	0.79-2.28	
						Avg:	0.52	0.03-2.28	
	Current study	EIA	LE	6-8	0.46 ± 0.36	0.86 ± 0.23	0.54	-0.22-1.60	86%
E2 (pg/ml)	Döhler & Wuttke 1975	RIA, NIAMD	SD	8-15	142 ± 10	222 ± 51	0.64	0.45-1.07	
	Kamberi, et al., 1980	RIA	W	4	165 ± 21	200 ± 35	0.83	0.55-1.32	
	Konkle & McCarthy 2011	RIA, DSL	SD	6	17 ± 0.038	19 ± 0.0014	0.89	0.89-0.90	
						Avg:	0.79	0.45-1.32	
	Current study	EIA	LE	6-8	239.5 ± 82.1	765.7 ± 289.1	0.31	0.11-1.04	63%
P4 (ng/ml)	Kamberi, et al., 1980	RIA	W	4	3.2 ± 0.3	2.2 ± 0.83	1.46	0.83-5.47	
	Döhler & Wuttke 1974	RIA, NIAMD	SD	6	0.88 ± 0.071	1.1 ± 0.26	0.80	0.54-1.42	
	Döhler & Wuttke 1975	RIA, NIAMD	SD	8-15	2.8 ± 5.5	2.9 ± 0.48	0.97	-2.36-4.46	
						Avg:	1.073	-2.36-5.47	
	Current study	EIA	LE	6-8	0.32 ± 0.16	0.36 ± 0.10	0.89	0.10-2.26	100%
T (ng/ml)	Döhler & Wuttke 1975	RIA, NIAMD	SD	8-15	0.64 ± 0.22	0.51 ± 0.24	1.26	0.43-6.92	
	Forest 1979	RIA	SD	6-8	0.19 ± 0.012	0.089 ± 0	2.13	N.E.	
						Avg:	1.69	N.E.	
	Current study	EIA	LE	6-8	0.50 ± 0.14	0.23 ± 0.10	2.17	0.89-9.99	66%

Male and female values represent means ± SEM extracted from original papers. Ratios (M:F) were calculated from means and SEMs using an online GraphPad program (http://www.graphpad.com/quickcalcs/errorProp1/). "Current study" = the control group (BPA 0) of this study, "antibody" = organization that provided antibodies, "RIA" = radioimmunoassay, "EIA" = enzyme-linked immunosorbent assay (ELISA), "SD" = Sprague-Dawley, "W" = Wistar, "LE" = Long-Evans, "n" = number of pups, "N.E." = not estimable due to zero in denominator.

References

Akingbemi, B. T., Sottas, C. M., Koulova, A. I., Klinefelter, et al., (2004). Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. *Endocrinology*, *145*(2), 592-603.

Aloisi, A. M., Della Seta, D., Rendo, C., et al., (2002). Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. *Brain Research*, *937*(1), 1-7.

Andrews, K., Fitzgerald, M. (1997). Barriers to optimal pain management in infants, children, and adolescents: Biological barriers to paediatric pain management. *The Clinical Journal of Pain*, *13*(2), 138-143.

Andrews, W. W., Advis, J. P., Ojeda, S. R. (1981). The maturation of estradiol-negative feedback in female rats: Evidence that the resetting of the hypothalamic "gonadostat" does not precede the first preovulatory surge of gonadotropins. *Endocrinology*, *109*(6), 2022-2031.

Bai, Y., Chang, F., Zhou, R., et al., (2011). Increase of anteroventral periventricular kisspeptin neurons and generation of E2-induced LH-surge system in male rats exposed perinatally to environmental dose of bisphenol-A. *Endocrinology*, *152*(4), 1562-1571.

Box, G. E. P. (1949). A general distribution theory for a class of likelihood criteria. *Biometrika,* **36**: 317–346.

Calabrese, E. J., Baldwin, L. A. (2001). U-shaped Dose-responses in biology, toxicology, and public health 1. *Annual Review of Public Health*, *22*(1), 15-33.

Chen, F., Zhou, L., Bai, Y., et al., (2014). Sex differences in the adult HPA axis and affective behaviors are altered by perinatal exposure to a low dose of bisphenol A. *Brain Research*, *1571*, 12-24.

Clancy, B., Finlay, B. L., Darlington, R. B., et al., (2007). Extrapolating brain development from experimental species to humans. *Neurotoxicology*, *28*(5), 931-937.

Dahl, K. D., Jia, X. C., Hsueh, J. W. (1988). Bioactive follicle-stimulating hormone levels in serum and urine of male and female rats from birth to prepubertal period. *Biology of Reproduction*, *39*(1), 32-38.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., et al., (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293-342.

Dobbing, J., Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Human Development*, *3*(1), 79-83.

Doerge, D. R., Vanlandingham, M., Twaddle, N. C., et al., (2010). Lactational transfer of bisphenol A in Sprague–Dawley rats. *Toxicology Letters*, *199*(3), 372-376.

Durando, M., Kass, L., Perdomo, V. (2011). Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats. *The Journal of Steroid Biochemistry and Molecular Biology*, *127*(1), 35-43.

Döhler, K. D., Wuttke, W. (1974). Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats. *Endocrinology*, *94*(4), 1003-1008.

Döhler, K. D., Wuttke, W. (1975). Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats. *Endocrinology*, *97*(4), 898-907.

Ferguson, S. A., Law, C. D., Abshire, J. S. (2011). Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures. *Toxicological Sciences*, 124(1), 149-160.

Ferguson, S. A., Law, C. D., Kissling, G. E. (2014). Developmental treatment with ethinyl estradiol, but not bisphenol A, causes alterations in sexually dimorphic behaviors in male and female Sprague Dawley rats. *Toxicological Sciences*, 140(2), 375-392.

Forest, M. G. (1979). Plasma androgens (testosterone and 4-androstenedione) and 17-hydroxyprogesterone in the neonatal, prepubertal and peripubertal periods in the human and the rat: differences between species. *Journal of Steroid Biochemistry*, *11*(1), 543-548.

Gámez, J. M., Penalba, R., Cardoso, N., et al., (2015). Exposure to a low dose of bisphenol A impairs pituitary-ovarian axis in prepubertal rats: effects on early folliculogenesis. *Environmental Toxicology and Pharmacology*, *39*(1), 9-15.

Gámez, J. M., Penalba, R., Cardoso, N., et al., (2014). Low dose of bisphenol A impairs the reproductive axis of prepuberal male rats. *Journal of Physiology and Biochemistry*, *70*(1), 239-246.

Heindel, J. J. (2005). The fetal basis of adult disease: role of environmental exposures—introduction. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *73*(3), 131-132.

Henze, N., Zirkler, B. (1990). A class of invariant consistent tests for multivariate normality. *Communications in Statistics: Theory and Methods* 19(10), 3595-3618.

Howdeshell, K. L., Furr, J., Lambright, C. R., et al., (2008). Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male Long Evans hooded rat. *Toxicological Sciences*, *102*(2), 371-382.

John, J. A., Draper, N. R. (1980). An alternative family of transformations. *Applied Statistics*, 29(2), 190-197.

Kamberi, I. A., De Vellis, J., Bacleon, E. S., et al., (1980). Hormonal patterns of the hypothalamo-pituitary-gonadal axis in the rat during postnatal development and sexual maturation. *Endokrinologie*, *75*(2), 129-140.

Kato, H., Furuhashi, T., Tanaka, M., et al., (2006). Effects of bisphenol A given neonatally on reproductive functions of male rats. *Reproductive Toxicology*, *22*(1), 20-29.

Kobayashi, K., Kubota, H., Ohtani, K., et al., (2012). Lack of effects for dietary exposure of bisphenol A during in utero and lactational periods on reproductive development in rat offspring. *The Journal of Toxicological Sciences*, *37*(3), 565-573.

Konkle, A. T., McCarthy, M. M. (2011). Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. *Endocrinology*, *152*(1), 223-235.

Mardia, K. V. (1970). Measures of multivariate skewness and kurtosis with applications. *Biometrika* 57(3), 519-530.

Melnick, R., Lucier, G., Wolfe, M., et al., (2002). Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environmental Health Perspectives* 110(4), 427-431.

Monje, L., Varayoud, J., Luque, E. H., et al., (2007). Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor α transcripts with alternative 5'-untranslated regions in the female rat preoptic area. *Journal of Endocrinology*, *194*(1), 201-212.

Nanjappa, M. K., Simon, L., Akingbemi, B. T. (2012). The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. *Biology of Reproduction*, *86*(5), 135.

Ojeda, S. R., Ramirez, V. D. (1972). Plasma level of LH and FSH in maturing rats: response to hemigonadectomy. *Endocrinology*, *90*(2), 466-472.

Prevot, V. (2015). Puberty in mice and rats. *Knobil and Neill's Physiology of Reproduction. New York, Elsevier*, 1395-1439.

Ramos, J. G., Varayoud, J., Kass, L., et al., (2003). Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. *Endocrinology*, *144*(7), 3206-3215.

Sadowski, R. N., Park, P., Neese, S. L., Ferguson, D. C., et al., (2014). Effects of perinatal bisphenol A exposure during early development on radial arm maze behavior in adult male and female rats. *Neurotoxicology and Teratology*, *42*, 17-24.

Tate, J., Ward, G. (2004). Interferences in immunoassay. *Clinical Biochemistry Reviews*, *25*(2), 105-120.

Taylor, J. A., Welshons, W. V., Vom Saal, F. S. (2008). No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24h after administration in neonatal female mice. *Reproductive Toxicology*, *25*(2), 169-176.

Taylor, J.A., Vom Saal, F.S., Welshons, et al., (2011). Similarity of bisphenol A pharmacokinetics in rhesus monkeys and mice: relevance for human exposure. *Environmental Health Perspectives*. 119(4), 422-430.

Wadhwa PD, Buss C, Entringer S, et al., (2009) Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. *Seminars in Reproductive Medicine*. 27(5), 358-368.

Vandenberg, L. N., Ehrlich, S., Belcher, S. M., et al., (2013). Low dose effects of bisphenol A: An integrated review of in vitro, laboratory animal, and epidemiology studies. *Endocrine Disruptors*, *1*(1), e26490.

Vom Saal, F. S., Akingbemi, B. T., Belcher, S. M., et al., (2007). Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. *Reproductive Toxicology*, *24*(2), 131-138.

Overall Thesis Conclusion

Strain differences in steroidal sex hormone profiles were found between three popular outbred strains of rat, Sprague-Dawley, Long-Evans, and Wistar. The strain differences were dependent on the sex and age of the animal, and likely extend to other reproductive endpoints in a complex manner. These differences between strains in the profiles of sex steroid hormones may point to inherent differences in the mechanisms of homeostatic maintenance of hormone levels, and/or susceptibility to endocrine disruptors, that could at least partially explain the issues in the inability to cross compare the effects seen in one strain versus another, leading to the lack of reproducibility and consistency of relevant-dose effects. However, additional studies of specific genetic products and loci involved in these differences in baseline hormone levels and responsiveness to endocrine-disrupting chemicals need to be conducted. Nevertheless, strain choice is certainly a factor amongst other experimental conditions that needs to be considered when conducting an animal study.

The second aspect of this thesis examined the effects of developmental exposure to relevant-dose perinatal BPA on still-developing Long-Evans rats. In this study, we found that BPA produced significant alterations on hormone profiles of neither gonadotropins nor sex steroids. This finding was consistent with some studies with similar experimental design, but also inconsistent with others. The study further supports the notion that strain choice may play a part in results obtained in a toxicological study, as it neither negates nor agrees with the majority of prior study outcomes. Other factors such as stage of development are also likely to affect the observed results of a study.

In conclusion, consideration for basic scientific concepts in endocrine, reproductive, and metabolic physiology, genetic variation within or between species, and developmental dynamics all govern the outcome of experimental studies on reproductive or endocrine alteration. Thus, researchers must assure that they have adequate understanding of these systems before making conclusive resolutions on controversial chemicals such as BPA. Synthesizing such discoveries from even the most basic physiology still expands the present scope of knowledge and provides vital clues in the ongoing struggle to elucidate the mechanisms of reproductive toxicology.

Appendix

Table 20. Effects of sex on mean estimated hormone concentration by treatment (BPA 0, BPA 40, BPA 400).

Differences of LSMs (Treatment x Sex)

Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
BPA 0	0.21	0.069	20	3.1	0.0058
BPA 40	0.20	0.057	20	3.4	0.0027
BPA 400	0.14	0.054	20	2.5	0.022

Table 21. Effects of sex (male vs. female) on mean estimated hormone concentration by hormone (E2, P4, T) and treatment (BPA 0, BPA 40, BPA 400)

Differences of LSMs (Hormone x Treatment x Sex)

Hormone	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
E2	BPA 0	0.031	0.15	80	0.20	0.84
	BPA 40	-0.00094	0.13	80	-0.01	0.99
	BPA 400	0.019	0.12	80	0.16	0.87
P4	BPA 0	-0.051	0.15	80	-0.33	0.74
	BPA 40	-0.026	0.13	80	-0.21	0.84
	BPA 400	-0.059	0.12	80	-0.48	0.63
T	BPA 0	-0.056	0.15	80	-0.36	0.72
	BPA 40	-0.025	0.13	80	-0.20	0.84
	BPA 400	-0.061	0.12	80	-0.50	0.62

Table 22. Effects of treatment (BPA 0, 40, 400; A vs. B) on mean estimated hormone concentration by sex

Differences of LSMs (Treatment x Sex)

Sex	Treatment A	Treatment B	Estimate	Standard Error	DF	t Value	Pr > |t|
Female	BPA 0	BPA 40	0.028	0.026	20	1.1	0.30
	BPA 40	BPA 400	0.0015	0.025	20	0.06	0.95
	BPA 0	BPA 400	0.026	0.026	20	1.0	0.32
Male	BPA 0	BPA 40	0.045	0.086	20	0.53	0.60
	BPA 40	BPA 400	-0.059	0.075	20	-0.79	0.44
	BPA 0	BPA 400	0.10	0.084	20	1.2	0.23

Table 23. Effects of treatment (BPA 0, 40, 400; A vs. B) on mean estimated hormone concentration by hormone

Differences of LSMs (Hormone x Treatment)

Hormone	Treatment A	Treatment B	Estimate	Standard Error	DF	t Value	Pr > |t|
FSH	BPA 0	BPA 40	0.044	0.10	100	0.44	0.66
	BPA 40	BPA 400	-0.014	0.088	100	-0.16	0.87
	BPA 0	BPA 400	0.058	0.098	100	0.59	0.55
LH	BPA 0	BPA 40	0.099	0.10	100	0.99	0.33
	BPA 40	BPA 400	-0.11	0.088	100	-1.29	0.20
	BPA 0	BPA 400	0.21	0.098	100	2.16	0.033
E2	BPA 0	BPA 40	0.065	0.10	100	0.65	0.52
	BPA 40	BPA 400	0.018	0.088	100	0.21	0.83
	BPA 0	BPA 400	0.046	0.098	100	0.47	0.64
P4	BPA 0	BPA 40	-0.014	0.10	100	-0.14	0.89
	BPA 40	BPA 400	-0.017	0.088	100	-0.19	0.85
	BPA 0	BPA 400	0.0025	0.098	100	0.03	0.98
T	BPA 0	BPA 40	-0.011	0.10	100	-0.11	0.91
	BPA 40	BPA 400	-0.017	0.088	100	-0.20	0.84
	BPA 0	BPA 400	0.0065	0.098	100	0.07	0.95

Table 24. Effects of treatment (BPA 0, 40, 400; A vs. B) on mean estimated hormone concentration by sex and hormone

Differences of LSMs (Hormone x Treatment x Sex)

Sex	Hormone	Treatment A	Treatment B	Estimate	Standard Error	DF	t Value	Pr > |t|
Female	FSH	BPA 0	BPA 40	0.038	0.058	80	0.66	0.51
		BPA 40	BPA 400	-0.0031	0.056	80	-0.05	0.96
		BPA 0	BPA 400	0.041	0.058	80	0.71	0.48
	LH	BPA 0	BPA 40	0.049	0.058	80	0.85	0.40
		BPA 40	BPA 400	0.0024	0.056	80	0.04	0.97
		BPA 0	BPA 400	0.047	0.058	80	0.80	0.42
	E2	BPA 0	BPA 40	0.049	0.058	80	0.84	0.40
		BPA 40	BPA 400	0.0083	0.056	80	0.15	0.88
		BPA 0	BPA 400	0.040	0.058	80	0.70	0.49
	P4	BPA 0	BPA 40	-0.0016	0.058	80	-0.03	0.98
		BPA 40	BPA 400	-0.00037	0.056	80	-0.01	0.99
		BPA 0	BPA 400	-0.0012	0.058	80	-0.02	0.98
	T	BPA 0	BPA 40	0.0043	0.058	80	0.07	0.94
		BPA 40	BPA 400	0.00050	0.056	80	0.01	0.99
		BPA 0	BPA 400	0.0038	0.058	80	0.06	0.95
Male	FSH	BPA 0	BPA 40	0.050	0.19	80	0.26	0.79
		BPA 40	BPA 400	-0.025	0.17	80	-0.15	0.88
		BPA 0	BPA 400	0.075	0.19	80	0.40	0.69
	LH	BPA 0	BPA 40	0.15	0.19	80	0.77	0.44
		BPA 40	BPA 400	-0.23	0.17	80	-1.37	0.17
		BPA 0	BPA 400	0.38	0.19	80	2.01	0.048
	E2	BPA 0	BPA 40	0.081	0.19	80	0.42	0.67
		BPA 40	BPA 400	0.029	0.17	80	0.17	0.86
		BPA 0	BPA 400	0.052	0.19	80	0.28	0.78
	P4	BPA 0	BPA 40	-0.027	0.19	80	-0.14	0.89
		BPA 40	BPA 400	-0.033	0.17	80	-0.20	0.84
		BPA 0	BPA 400	0.0062	0.19	80	0.03	0.97
	T	BPA 0	BPA 40	-0.026	0.19	80	-0.14	0.89
		BPA 40	BPA 400	-0.035	0.17	80	-0.21	0.83
		BPA 0	BPA 400	0.0092	0.19	80	0.05	0.96